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(19) (CA) APPLICATION FOR CANADIAN PATENT (12)

(54) Method of Treating Autoimmune Diseases

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Notice: This application is as filed and may therefore contain an incomplete specification.

ABSTRACT OF THE DISCLOSURE

A method of treating autoimmune diseases associated with a predominance of T cells expressing Vβ8$^+$ T cell receptor comprising administering am amount of Staphylococcus enterotoxin B, a derivative, analogue or active fragment thereof, effective to reduce the number and/or inactivate T cells expressing Vβ8$^+$ T cell receptor whereby there is a decrease in disease activity.

Title: METHOD OF TREATING AUTOIMMUNE DISEASES

FIELD OF THE INVENTION

The present invention relates to the use of Staphylococcus enterotoxin B to treat autoimmune diseases; to pharmaceutical compositions containing Staphylococcus enterotoxin B; and, to methods of treating autoimmune diseases using Staphlyococcus enterotoxin B.

BACKGROUND OF THE INVENTION

Superantigens (SuperAgs) (Marrack, P. and J. Kappler, Science (Wash. DC) 248:705, 1990) are molecules that in association with class II MHC activate T cells based solely on the Vβ chain of the TCR (Marrack, P. and J. Kappler, Science (Wash. DC) 248:705, 1990; Janeway et al. Immunol. Rev. 107:61, 1989). These antigens are the most powerful polyclonal mitogens known, stimulating a large proportion of both murine and human T cells. The minor lymphocyte stimulating (Mls) antigen, a self-SuperAg, has recently been reported to be encoded by a retrovirus, but the Mls antigen has yet to be isolated (Janeway, C.A., Nature (Lond.) 349:459, 1991; Marrack et al., Nature (Lond.) 349:524, 1991; Frankel et al., Nature (Lond.) 349:526, 1991; Woodland et al., Nature (Lond.) 349:529, 1991; Dyson et al., Nature (Lond.) 349:531, 1991). More is known about the bacterial SuperAgs, particularly the Staphylococcus aureus enterotoxin series (SEs), whose sequence and structure have been documented (Marrack P. and J. Kappler, Science (Wash. DC) 248:705, 1990). SuperAgs are known to exert powerful effects on the developing TCR repertoire in both $CD4^+$ and $CD8^+$ T cells (White et al., Cell. 56:27, 1989; Kappler et al., Nature (Lond.) 332:35, 1988; MacDonald et al., Nature (Lond.) 332:40, 1988; Fry, A. and L. Matis, Nature (Lond.) 335:830, 1988). Previous data from the inventor's laboratory (Kawabe, Y. and A. Ochi, J. Exp. Med. 172:1065, 1990; Kawabe, Y. and A. Ochi, Nature (Lond.) 349:245, 1991) have demonstrated both clonal anergy and deletion by

programmed cell death of peripheral T cells bearing reactive Vβ8 TCR with in vivo administration of one of these SuperAgs, Staphylococcus Enterotoxin B (SEB), which engages Vβ7 and 8-bearing T cells (Janeway et al., Immunol. Rev. 107:61, 1989; White et al., Cell. 56:27, 1989).

Recent work on different animal models of disease has demonstrated a Vβ predominance of T cells involved in disease pathogenesis. Examples of this phenomenon include experimental allergic encephalomyelitis (EAE) in B10.PL mice (Zamvil et al., J. Exp. Med. 167:1586, 1988) and lupus nephritis in MRL/lpr (Singer et al., Proc. Natl. Acad. Sci. USA 83:7018, 1986), both of which show a Vβ8 restriction in T cells. Specific immunotherapies aimed at intervention at the level of these pathogenic T cells have included anti-Vβ8 antibody (Ab) (Zaller eta l., J. Exp. Med. 171:1943, 1990) and Vβ8 peptide therapy (Vandenbark et al., Nature (Lond.) 341:541, 1989) in EAE, and anti-T cell (Thy-1.2 [Wofsy et al., J. Immunol. 134:852, 1985], CD4 [Santoro et al., J. Exp. Med. 167:1713, 1988], B220 [Asensi et al., Immunology 68:204, 1989]) Ab treatment in MRL/lpr mice. These treatments have resulted in both the reduction in the number of "targeted" T cells and a corresponding improvement in the disease state. However, the effects of passive antibody therapy are generally short-lived and require frequent injections of large amounts to be effective.

$CD4^-CD8^-$ "double-negative" T cells ((DN) T cells) have been implicated in disease initiation and/or pathogenesis. It has been shown that these cells have undergone thymic selection and expression of surface CD4 and CD8 (Kotzin B.L. et al J. Exp. Med. 168:2221 and Mountz, T.D. et al, J. Immunol. 144:2159). There are currently two main paradigms as to the origin of these DN T cells (Singer, P.A. and A.N. Theofilopoulos, Immunol. Rev. 118:103). They

may encode TCRs with autoreactive specificities or they may arise consequent to defective positive selection and may not have specificity for self at all. Regardless of their origins DN T cells are clearly involved in disease pathogenesis.

SUMMARY OF THE INVENTION

The present inventor has significantly found that administration of Staphylococcus aureus enterotoxin B (SEB) in vivo dramatically suppresses disease activity in autoimmune mice. In particular, SEB treatment in MRL/lpr mice resulted in a reduction in Vβ8$^+$, CD4$^-$CD8$^-$ peripheral T cells concomitant with disease suppression. Clinical and serological disease activity was reduced in SEB-treated mice in a dose-dependent manner. Disease activity was also found to be dramatically reduced in autoimmune NZBxNZWF1 mice which display clinical symptoms comparable to those found with human autoimmune diseases.

Therefore, the present invention relates to a method of treating autoimmune diseases associated with a predominance of T cells expressing Vβ8$^+$ T cell receptor comprising administering an amount of Staphylococcus enterotoxin B, a derivative, analogue or active fragment thereof, effective to inactivate and/or reduce the number of T cells expressing Vβ8$^+$ T cell receptor whereby there is a decrease in disease activity.

The invention also relates to a method of using Staphylococcus enterotoxin B, derivatives, analogues or fragments thereof to assay for T cells associated with autoimmune disease pathogenesis in a sample. Preferably, T cells expressing Vβ8$^+$ T cell receptor (TCR), most preferably, Vβ8$^+$ CD4$^-$CD8$^-$ TCR are assayed.

Further, the invention relates to a method of using Staphylococcus aureus enterotoxin B, derivatives,

analogues or fragments thereof to down regulate lymphokines, preferably tumor necrosis factor and/or IL6.

BRIEF DESCRIPTION OF THE DRAWINGS

Further details of the invention are described below with the help of the examples illustrated in the accompanying drawings in which:

Figure 1 shows circulating immune complex and anti-DNA titers as measured by ELISA;

Figure 2 shows a flow cytometric analysis of MRL/lpr spleen and lymph node cells;

Figure 3 shows the mass of lymphoid organs from MLR/lpr mice;

Figure 4 shows the apoptosis of spleen T cells of SEB or TSST-1 injected mice;

Figure 5 shows SEB-specific spleen cell response on day 10;

Figure 6 shows SEB-specific spleen cell response on 2 months;

Figure 7 shows SEB-specific spleen cell response on 4 months;

Figure 8 shows the kinetics of SEB-response recovery and $V\beta8^+$ cell proportion in non-anergic (Thy1.1) and anergic (Thy1.2) chimeric mice;

Figure 9 shows that anti-$V\beta8$ antibody treatment fails to generate inositol phosphates in SEB-anergized spleen T cell, but co-stimulation by PMA plus ionomycin induces IL-2 production in anergic $V\beta8^+$ T cells;

Figure 10 shows that failure to increase intra-cellular protein tyrosine phosphorylation in SEB-anergized spleen T cell with SEB or anti-$V\beta8$ antibody treatments;

Figure 11 shows an analysis of $V\beta8$-TCR-associated, CD3-ζ in anergic spleen T cells;

Figure 12 shows phosphatase activity of anergic $V\beta8^+$ T cells;

Figure 13 shows an analysis of CD3-ζ and *fyn* proteins, and *in vitro* kinase function in $V\beta8^+$ T cells;

Figure 14 shows growth inhibition of 38C13 B lymphoma by SEB-stimulated spleen cells (1a) and the pre-treatment of 38C13 B lymphoma with anti-Id-SEB inhibits the growth of 38C13 in co-culture with spleen cells (1b);

Figure 15 shows the effect of injection of targeting reagents on peripheral T cell function; and

Figure 16 shows the targeting of B lymphoma (Class II MHC^-, Id^+) with SEB-anti-Id; and

Figure 17 shows the results of reduction of serum anti-DNA antibody titer of autoimmune mice treated with a bacterial superantigen, SEB.

DETAILED DESCRIPTION OF THE INVENTION AND PREFERRED EMBODIMENTS

The present inventors investigated the effects of biweekly intravenous injections of Staphylococcus aureus Enterotoxin B (SEB) into autoimmune MRL-lpr/lpr (MRL/lpr) mice. Rather than causing the expansion of $V\beta8^+$ T cells, SEB administration resulted in the reduction of $V\beta8^+$, $CD4^-$ $CD8^-$ "double-negative" (DN) T cells. This was shown by FACS analysis as this putative pathogenic population was diminished in both spleen and lymph node. The symptoms of systemic lupus erythematosus (SLE) in MRL/lpr which include high titers of anti-DNA antibodies and circulating immune complexes and proteinuria were reduced in SEB-treated mice in a dose-dependent manner. The clinical parameters of SLE in MRL/lpr, which include lymph node hyperplasia and necrotic vasculitis, were suppressed in 50 µg SEB-treated mice. T cells bearing Vβ6 T cell receptor, which does not interact with SEB, were not reduced with SEB administration. Thus, disease suppression was associated with a specific reduction in the number of $V\beta8^+$, DN T cells. These results implicated a therapeutic role of superantigen-based immunotherapy in Vβ-restricted T cell dominated clinical syndromes.

The present inventor also found that SEB dramatically

reduced disease activity in NZBxNZWF1 mice. These mice demonstrate clinical symptoms comparable to those found with human autoimmune diseases (Theofilopoulos and FJ Dixon, Adv. Immunol. 37, (1985)).

The present inventor also found that there were differences among superantigens in their ability to induce T cell tolerance in vivo. SEA and SEB were found to induce tolerance in treated mice whereas injection of toxic shock syndrome toxins (TSST-1) does not result in tolerance. Therefore, previous observations which relate superantigens to the suppression of reactive Vβ TCR T cells seem difficult to generalize to all bacterial superantigens.

As hereinbefore mentioned, the present invention relates to a method of treating autoimmune diseases associated with a predominance of T cells expressing $V\beta8^{+}$ T cell receptor comprising administering an amount of Staphylococcus enterotoxin B, a derivative, analogue or active fragment thereof, effective to inactivate and/or reduce the number of T cells expressing $V\beta8^{+}$ T cell receptor whereby there is a decrease in disease activity.

Staphylococcus aureus enterotoxin B, preferably, may be used in the invention. The sequence of Staphylococcus aureus enterotoxin B is described by James C.& Khan S. J. Bacteriol. 166:29, (1986). Derivatives of Staphylococcus aureus enterotoxin B, preferably methylated, acylated, acetylated, etc. derivatives or conjugates with immunogens; analogues of Staphylococcus aureus enterotoxin B, preferably structural or functional analogues of Staphylococcus aureus enterotoxin B such as Staphylococcus enterotoxin B with additions, deletions or replacements of amino acids; or fragments of the Staphylococcus aureus enterotoxin B may also be used in the present invention. Derivatives, analogues and fragments which may be used in

the invention may be identified by their ability to bind to the $V\beta8^+$ T cell receptor (TCR), most preferably to $V\beta8^+$, $CD4^-$ $CD8^-$ "double-negative" (DN) T cells.

The Staphylococcus aureus enterotoxin B, derivatives, analogues or fragments thereof, used in the invention may be a naturally occurring protein. They may also be obtained by expression in a suitable host cell using techniques known in the art. Suitable host cells include prokaryotic or eukaryotic organisms or cell lines. The proteins may be prepared by chemical synthesis using techniques well known in the chemistry of proteins such as solid phase synthesis (Merrifield, 1964, J. Am. Chem. Assoc. 85:2149-2154) or synthesis in homogenous solution (Houbenweyl, 1987, Methods of Organic Chemistry, ed. E. Wansch, Vol. 15 I and II, Thieme, Stuttgart). The Staphylococcal aureus enterotoxin B may also be commercially obtained; for example, from Sigma Chemical Co., St. Louis, MO.

The in vivo administration of Staphylococcal aureus entertoxin B, derivatives, analogues or fragments thereof, results in inactivation and/or reduction of T cell populations associated with the pathogenesis of autoimmune diseases and a concomitant suppression of disease activity. Accordingly, Staphylococcus entertoxin B, derivatives, analogues or fragments thereof may be used for the treatment of autoimmune diseases, preferably autoimmune disease associated with a predominance of T cells expressing $V\beta8^+$ T cell receptor (TCR), most preferably expressing $V\beta8^+$, $CD4^-CD8^-$. In particular, Staphylococcus aureus entertoxin B, derivatives, analogues or fragments thereof may be used to treat autoimmune diseases including multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, Type I diabetes, insulin-dependent diabetes mellitus, myasthenia gravis and pemphigus vulgaris, AIDs, Lupus Nephritis, allergic encephalomyelitis.

Staphylococcus aureus entertoxin B, derivatives, analogues or fragments thereof, may be administered alone or together with other agents. The active substance may be formulated into a pharmaceutical composition for oral, topical, rectal, parenteral, local, inhalant or intracerebral use. The pharmaceutical compositions may therefore be in solid or semisolid form, for example pills, tablets, creams, gelatin capsules, capsules, suppositories, soft gelatin capsules, gels, membranes, tubelets. For parenteral and intracerebral uses, those forms for intramuscular or subcutaneous administration can be used, or forms for infusion or intravenous or intracerebral injection can be used, and can therefore be prepared as solutions of the active substances or as powders of the active substances to be mixed with one or more pharmaceutically acceptable excipients or diluents, suitable for the aforesaid uses and with an osmolarity which is compatible with the physiological fluids. For local use, those preparations in the form of creams or ointments for topical use or in the form of sprays should be considered; for inhalant uses, preparations in the form of sprays, for example nose sprays, should be considered.

The pharmaceutical compositions can be intended for administration to humans or animals. They contain preferably between about 0.005 and 2 mg/ml of active component in the case of solutions, sprays, ointments and creams and preferably between about 0.005 and 2 mg/ml of active compound in the case of solid form preparations. Dosages to be administered depend on individual needs, on the desired immunosuppressive effect and on the chosen route of administration, but daily dosages to humans by subcutaneous, intramuscular or intracerebral injection generally vary between 0.5µg to 50 µg of active substance per kg of body weight.

The pharmaceutical compositions can be prepared by <u>per se</u>

known methods for the preparation of pharmaceutically acceptable compositions which can be administered to patients, and such that an effective quantity of the active substance is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example, in Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA 1985). On this basis, the pharmaceutical compositions include, albeit not exclusively, solutions of the solutions of the Staphylococcus entertoxin B, derivatives, analogues or fragments thereof in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered solutions with a suitable pH and iso-osmotic with the physiological fluids.

The pharmaceutical compositions may also contain or be used in conjunction with other agents such as antibodies to T cell markers, and immunosuppression agents.

Staphylococcus entertoxin B, derivatives, analogues or fragments thereof may be used to assay for T cells associated with autoimmune disease pathogenesis in a sample. Preferably T cells expressing $V\beta8^{+}$ ^{TCR}, most preferably $V\beta8^{+}$ $CD4^{-}CD8^{-}$ TCR are assayed. Thus, the Staphylococcus aureus entertoxin B, derivatives, analogues or fragments thereof may be used to diagnose autoimmune diseases.

Staphylococcus aureus entertoxin B has also been found to down regulate tumor necrosis factor and IL6. Accordingly, Staphylococcal aureus entertoxin B, derivatives, analogues or fragments thereof may also be used to down regulate lymphokines such as tumor necrosis factor and/or IL6.

The following non-limiting examples are illustrative of the present invention:

EXAMPLES

Example 1

The following materials and methods were used in the investigations outlined in Example 1:

Animals. MRL/Mp-lpr mice (4-6 week old) were purchased from The Jackson Laboratory (Bar Harbor, ME).

Reagents and mAbs. SEB was purchased from Sigma Chemical Co. (St. Louis, MO). Reagents used for direct staining were biotinconjugated anti-: Vβ6 (Kanagawa et al., Cell. Immunol. 119:412, 1989), Vβ8 (Staertz et al., J. Immunol.

134:3994, 1985), CD4-PE conjugate, and CD8-FITC conjugate from Becton Dickinson & Co. (Mountain View, CA). Secondary reagents, streptavidin-PE(SA-PE) and avidin-FITC, were also purchased from Becton Dickinson & Co.

Treatment of MRL/lpr with SEB. Treatment of MRL/lpr started at 6 weeks of age before clinical onset of disease. Animals were injected with 50, 5, and 0.5 µg of SEB in 0.2 ml PBS or PBS alone intravenously through the tail vein every 2 week.

Fluorescence Staining (FACS®). Spleen and axillary lymph node single cell suspensions from 6-mo-old MRL/lpr mice were treated with Tris-buffered 0.16 M ammonium chloride to lyse the RBC. 10^6 cells were incubated with biotinylated anti-Vβ6 or anti-Vβ8 (purified from the respective B cell hybridomas by protein A-Sepharose [Pharmacia Fine Chemicals, Uppsala, Sweden]) for 30 min. on ice. The cells were then washed and incubated with either SA-PE (when anti-CD8-FITC was used as the second antibody) or avidin-FITC (when anti-CD4-PE was used as the second antibody). After 30 min. of incubation on ice, the cells were washed three times and incubated with anti-CD4-PE or anti-CD8-FITC (as mentioned above). Cells were washed and two-color analysis was performed by an Epics C fluorocytometer (Coulter Immunology, Hialeah, FL).

ELISA for Anti-DNA Abs and Circulating Immune Complexes. Polystyrene microtiter wells coated with double-stranded DNA (ds-DNA) or goat C1q were the gift of Dr. Marilyn Baltz (Sigma Chemical Co.). Blood from each mouse was pooled according to treatment group and collected before the biweekly injections. Sera was diluted in 0.05% Tween 20 in PBS at a 1:500 dilution and allowed to incubate on the plates for 60 min. at room temperature. The plates were then washed three times with PBS-Tween, and 50 µl of a 1/1,000 dilution of anti-IgG and anti-IgM goat anti-

mouse urease conjugate (Sigma Chemical Co.) was added to the plates. After incubation for 30 min., the plates were washed three times with PBS-Tween and twice with 0.15 M NaCl. The plates were then incubated with the urease substrate solution. The urease substrate solution was made by a protocol kindly provided by Sigma Chemical Co. In short, 8 mg of bromcresol purple was dissolved in 1.48 ml of 0.01 M NaOH and then diluted to 100 ml with water. 100 mg of urea and 3.7 mg of EDTA were dissolved and the pH was adjusted to 4.8 by the addition of 0.01 M NaOH. Colorimetric change was quantified by a Microplate Reader (MR 600; Dynatech, Chantilly, VA) at 590 nm OD.

Proteinuria and Physical Symptoms. Urine (from at least four mice per group) was pooled according to treatment group. Protein concentration and the presence of blood in urine was measured semi-quantitatively by reagent strips for urine analysis (Labstrix; Ames Corp., Etobicoke, Ontario). Physical symptoms were visually scored as: 0, no symptoms; 0.5, trace; 1-4, when visible symptoms are observed, with 4 being the most severe (physical symptoms include lymph node hyperplasia, immune complex vasculitis, and necrosis of the ears). Mortality was observed in the PBS and 0.5-μg SEB-treated groups at 4 mo of age. The scores representing physical symptoms were calculated by determining the total score for each group and then dividing by the number of animals alive in that group when the measurement was taken.

A. Systemic Administration of SEB Reduces Both Immune Complex and dsDNA-specific Ab Titer.

Serologically, both circulating immune complex (Fig. 1a) and ds-DNA-specific antibody (Fig. 1b) titers were followed as measured by ELISA. Figure 1 shows circulating immune complex and anti-DNA titers as measured by ELISA. MRL-lpr/lpr mice were given biweekly intravenous

injections (by tail vein) of 50, 5, and 0.5 μg of SEB in 0.2 ml PBS or PBS alone beginning at 6 week of age. At weekly intervals, ~200 μl of blood was collected from each mouse and serum was pooled according to treatment group and stored at -20°C. (a) The results from C1q-coated plates; (b) DNA-coated wells. The data are representative of three separate experiments. Nonautoimmune C3H/HeJ and pre-autoimmune MRL/lpr (4 week of age) registered OD readings <0.05 at 590 nm for both assays. 50 μg SEB (●), 5 μg SEB (O), 0.5 μG SEB (▵), PBS (□). The results show that while the PBS-injected controls manifested the aggressive increase in these indices correlated with disease onset at 3 mo of age, animals treated with 50 μg SEB showed a pre-autoimmune level, similar to that measured in nonautoimmune mice. The intermediate doses of SEB (5 and 0.5 μg) decreased both serological levels 1-1.5-fold, but did not have as great an effect as the 50-μg SEB treatment, which was associated with a fourfold difference in DNA-specific OD, and an eightfold difference in immune complex OD (from this point on, "SEB treatment" will refer to the 50-μg-treated group exclusively). A decrease in ds-DNA-specific Ab titer using 20μg of SEB has been prviously observed (unpublished observations). Thus, SEB treatment induces a dose-dependent decrease in two serological parameters of SLE in MRL/lpr mice.

B. Specific reduction of Vβ8$^+$, CD4$^-$CD8$^-$ T Cells in SEB-treated Spleens and Lymph Nodes.

To ascertain the effects of SEB immunization on the peripheral Vβ8 population in this autoimmune strain, the proportions of Vβ8$^+$ T cells in the splenic and lymph node CD4$^+$, CD8$^+$, and CD4$^-$CD8$^-$ T cell populations were compared between 6-mo-old controls and 50-μg SEB-treated mice. The investigation was carries out to determine if SEB could cause a decrease in peripheral Vβ8$^+$ T cells that were CD4$^-$ and CD8$^-$. Figure 2 shows the flow cytometric analysis of

MRL/lpr spleen and lymph node cells. MRL/lpr mice were treated with 50 μg SEB or PBS alone and were killed at 6 mo of age, and the spleens (a) and axillary lymph nodes (b) were removed. Single cell suspensions were analyzed by two-color FACS® analysis with biotinylated anti-Vβ6 and -Vβ8 Abs, and CD4-PE and CD8-FITC Abs. The results are presented as the mean and SD of three separate experiments. The results of both splenic and lymph node analyses show that injections of SEB does produce a decrease in the Vβ8$^+$ DN T cell fraction, from 15% to 2% in spleen (Fig. 2a) and 22% to 2% in LN (Fig. 2b). Interestingly, there seemed to a compensatory increase in the Vβ6$^+$ CD4$^-$8$^-$ fraction with SEB treatment, from 3% to 8%. However, the Vβ6$^+$ DN T cell proportion did not show the enormous expansion normally observed in the Vβ8$^+$ DN T cell fraction. Also, SEB immunization led to minimal decreases in the proportions of Vβ8$^+$ CD4$^+$ and Vβ8$^+$ CD8$^+$ T cells.

The results imply that the abnormal DN T cells that are associated with both onset and acceleration of disease in the MRL/lpr strain are more susceptible to deletion than single-positive cells, and/or that SEB treatment has suppressed their time-dependent proliferation. Regardless, the results show that SEB treatment has decreased the level of these phenotypically aberrant cells and results in the suppression of disease.

C. Mass of Lymphoid Organs from SEB-treated MRL/lpr Mice.

One of the phenotypic changes associated with disease onset is a massive lymphoid hyperplasia, which causes a profound increase in the size of lymphoid organs (Andrews et al., J. Exp. Med. 148:1198, 1978; Theofilopoulos, A.N. and F.J. Dixon, Adv. Immunol. 37:269, 1985). Therefore the splenic and lymph node sizes of mice from PBS controls and SEB-treated MRL/lpr were examined.

Figure 3 shows the mass of lymphoid organs from MLR/lpr mice. Spleen and axillary lymph nodes were removed from 6-mo-old MRL/lpr mice treated with 50 µg SEB or PBS and weighed. The data are presented as the mean mass per spleen or axillary lymph node and SD of four different experiments.

As shown in Figure 3, with SEB treatment, there is a decrease in both the spleen and the axillary lymph node. Specifically, spleens and axillary lymph nodes from PBS controls averaged 0.66 and 0.25 g, respectively. The SEB counterparts weighed 0.25 and 0.04 g. This decrease in splenic and axillary lymph node mass is due to a decrease in cell number in these lymphoid organs. The other lymph nodes demonstrated a similar decrease in cellularity and size with SEB treatment (data not shown).

D. Reduction in Proteinuria and the Physical Symptoms of SLE.

Disease onset in these mice occurs at 3 mo of age with visible lymphoid hyperplasia, increasing titers of auto-Abs, and the development of nephritis with proteinuria. 50% mortality in the MRL/lpr strain, due to glomerulonephritis, occurs by 5 mo of age. To determine the effect of SEB treatment on a clinical level, renal function was measured by assaying proteinuria and the physical symptoms of disease were followed.

In Table 1, it is clearly shown that SEB treatment has inhibited renal degradation (0.3 g/liter at 5 mo of age), while the PBS controls demonstrate a gross defect in renal function (3.0 g/liter). The 0.5-µg SEB-treated group shows an apparent acceleration of renal degradation, whereas the 5-µg SEB-treated group reaches abnormal levels at 5 mo of age. In fact, by 21 weeks of age, all the treatment groups, except 50-µg SEB, show high levels of

proteinuria as well as the presence of blood in the urine, a further indicator of glomerulonephritis.

The visible physical symptoms that signal lupus in this strain include lymph node hyperplasia, necrosis of the ears, and immune complex vasculitis of the skin (Andrews et al. J. Exp. Med. 148:1198, 1978; Theofilopoulos, A.N. and F.J. Dixon, Adv. Immunol. 37:269, 1985). Table 2 shows that SEB treatment has been effective in suppressing the physical signs of disease and parallels the results of SEB treatment on the other parameters of SLE in the MRL/lpr strain.

<u>Example 2</u>

This example deals with the heterogeneous effects on T cells caused *in vivo* by Staphylococcal enterotoxins (SE's) A distinction is proposed among the members of the bacterial superantigens formally depending on their ability to induce tolerance in specific Vβ positive T cells.

A. <u>Molecular homology of bacterial superantigens</u>

Although the molecular biochemical structure of MMTV encoded superantigens is not yet clear, that of SE's has been well studied. They are single chain molecules with molecular weight 23-30 kDa. The amino acid and DNA-sequences of each superantigen revealed that they are closely related family members. Based upon the serological studies, SEs are classified as SEA, B, C1, C2, C3, D, E, and TSST-1. SEA and SEE are closely related, having more than 90 % amino sequence conservation. SEB and SEC1 are 60 % alike in amino sequence, while SEA has 30 % conserved sequence with either SEB or SEC1 (Janeway Jr CA et al (1989) Immunological Reviews 107:61-88) and MarrackP. and Kappler J (1990) Science 248:705-711). Whereas the amino acid sequence of TSST-1 satisfies the required homology to be in this family, it is more related

to Streptococcal pyrogenic exotoxins than to other members (Schlievert PM (1983) J Infect Dis 147:391-398). Reflecting the homology of amino acid sequences, some antigenic determinants were also shared among SEs. SEs have a centrally located disulfide loop which is required for T cell activation. However TSST-1 does not possess a disulfide loop (Spero L. et al (1973) J Biol Chem 248:7289-7294.

B. Binding of SE's to class II MHC

Intracellular processing, which is required by most conventional protein antigens, is not necessary for superantigens to associate with class II MHC and stimulate T cells because native SE's can bind to class II MHC (Fraser JD (1989) Nature 339:221-223; Fischer H, et al (1989) J Immunol 142:3151-3157; Mollick JA, et al (1989) Science 244:817-821; and Scholl P, et al (1989) Proc Natl Acad Sci USA 86:4210-4214). They associate with class II MHC at the outside of the α-helix which creates an antigen-binding cleft (Dellabona P, et al (1990) Cell 62:1115-1121). Some SEs have higher affinity to class II MHC than others (eg. SEA > SEB > TSST-1) and it was suggested that the potency of stimulation of T cells is related to their affinity for class II MHC molecules (Mollick JA, et al (1991) J Immunol 146:463-468). Some SEs showed preferential binding to different class II MHC species in mice such that SEA and SEB equally bind to I-E and I-A but TSST-1 has more affinity for I-A (Mollick JA, et al (1991) J Immunol 146:463-468). In general, human HLA-DR has more affinity to bacterial superantigens than mouse class II MHC (Marrack P, Kappler J (1990) Science 248:705-711). Of interest is that the binding site of SEA and SEB to mouse class II MHC is different from that of TSST-1 (Fraser JD (1989) Nature 339:221-223; Fischer H, et al (1989) J Immunol 142:3151-3157; Mollick JA, et al (1989) Science 244:817-821; Scholl P, et al (1989) Proc

Natl Acad Sci USA 86:4210-4214; Herrmann T, et al (1989) Eur J Immunol 19:2171-2174; amd Pontzer CH, et al (1991) Proc Natl Acad Sci USA 88:125-128). SE's binding to class II MHC is known to lead to the generation of an intracellular signal in antigen presenting cells (APC's) like B cells and monocytes (Mourad W, et al (1989) J Exp Med 170:2011-2022 and Fuleihan R, et al (1991) J Immunol 146:1661-1666). This "activation" of APC triggers the synthesis of lymphokines such as interleukin-1 (IL-1)β and tumor necrosis factor (TNF)-α. Thus the differences in class II MHC-type, and binding affinity, binding sites, are critical factors for the activation of T cells and probably the APCs by SEs.

C. TCR Vβ-specific T cell stimulation

While all SEs stimulate T cells in the presence of class II MHC, SEs are highly heterogeneous with regard to the particular Vβ bearing T cells that they stimulate. Each SE selects a different set of Vβ expressing cells such that SEA stimulates Vβ1, 3, 10, 11, 12, and 17 T cells while SEB stimulates those expressing Vβ7 and 8.1,2,3 TCR (Herman A, et al (1991) Annu Rev Immunol 9:745-772). Since mice have only about 20 different Vβ regions available for use, each SE that stimulates multiple Vβ families activates a large number of T cells, likely more than 10 %. However, detailed study of $Mls-1^a$ reactive T cells has shown that a hierarchy exists among reactive $V\beta^+$ T cells with respect to their responsiveness (Waanders GA, and McDonald HR (1992) Eur J Immunol 22:291-29328). It was also indicated that the usage of different Vα families influences recognition of $MLs-1^a$ by $V\beta6^+$ T cells (Vacchio MS, et al(1992) J Exp Med 175:1405-1408). These reports suggest that the expression of particular Vβ is not the only factor but that more TCR components are involved in the determination of whether a T cell will react to superantigens.

D. In vitro and *in vivo* effects of bacterial superantigens on T cells.

The strong activation of T cells expressing limited Vβ's TCRs by SE's has been studied *in vitro* in mouse and human. The activation of such T cells induces the production of lymphokines such as interleukin-2 (IL-2), gamma-interferon (γIFN), TNF and lymphotoxins (Carlsson R, Sjögren H-O (1985) Cell Immunol 96:175-183 and Fisher H, et al (1990) J Immunol 144:4663-4669). Both CD4 and CD8 subsets proliferate in response to SEB (Kawabe Y, Ochi A (1990) J Exp Med 172:1065-1070 and Herrmann T, et al (1990) J Immunol 144:1181-1186). In contrast to CD4 T cells, which require a signal from CD4 molecules, CD8 T cell activation by SEB is independent of CD8 molecules (Herrmann T, et al (1990) J Immunol 144:1181-1186). Activated T cells exert cytotoxicity to SEB presenting class II MHC$^+$ B lymphoma target cells (Kawabe Y, Ochi A (1990) J Exp Med 172:1065-1070). Cytotoxicity was observed in CD4 T cells but not in CD8 T cells when fresh mouse spleen T cells were stimulated (Kawabe Y, Ochi A (1990) J Exp Med 172:1065-1070). Lymphokine activated killer (LAK) cell like cytotoxicity was also observed in SEA activated human peripheral blood mononuclear cells (Lando PA, et al(1991) Cancer Immunol Immunother 33:231-237).

In vivo effects of bacterial superantigens to T cells have been best studied using SEB. In neonatal mice injected with SEB every other day, immature and mature Vβ8$^+$ T cells were deleted in both CD4 and CD8 subsets in thymus (White J, Herman A, Pullen AM, Kubo R, Kappler JW, Marrack P (1989) Cell 56:27-35). When adult mice were SEB-primed by intravenous (i.v.), intraperitoneal (i.p.) or subcutaneous routes, the yield of thymocytes decreased (Marrack P, Blackman M, Kushnir E, Kappler J (1990) J Exp Med 171:455-464). However, the proportion of Vβ8$^+$ T cells in the periphery was elevated to approximately twice that of

normal mice by day 3 after injection (Kawabe Y, Ochi A (1991) Nature 349:245-248; MacDonald HR, Baschieri S, Lees RK (1991) Eur J Immunol 21:1963-1966; Newell KA, Ellenhorn JD, Bruce DS, Bluestone JA (1991) Proc Natl Acad Sci USA 88:1074-1078). This expansion of $V\beta8^+$ T cells in the periphery occurred in both CD4 and CD8 subsets. By the end of the first week the number of $V\beta8^+$ T cells declined to a level below normal in $CD4^+$ T cells (Kawabe Y, Ochi A (1991) Nature 349:245-248; and MacDonald HR, Baschieri S, Lees RK (1991) Eur J Immunol 21:1963-1966). The decline of $V\beta8^+$ T cells in the CD8 subset was less than that in the CD4 subset and returned to that of the normal animal (Kawabe Y, Ochi A (1991) Nature 349:245-248; and MacDonald HR, Baschieri S, Lees RK (1991) Eur J Immunol 21:1963-1966). In conjunction with the reduction of $V\beta8^+$ T cells *in vivo*, a study of DNA extracted from spleen T cells revealed they were undergoing programmed cell death (PCD) (Kawabe Y, Ochi A (1991) Nature 349:245-248). The expansion, reduction and PCD took place specifically in $V\beta8^+$ T cells but not in $V\beta6^+$ or $V\beta2^+$ T cells which do not react to SEB (Kawabe Y, Ochi A (1991) Nature 349:245-248; and MacDonald HR, Baschieri S, Lees RK (1991) Eur J Immunol 21:1963-1966). The induction of IL-2 receptor (IL-2R) expression on $V\beta8^+$, CD4 and CD8 T cells occurred within 18 hours after SEB injection (Newell KA, Ellenhorn JD, Bruce DS, Bluestone JA (1991) Proc Natl Acad Sci USA 88:1074-1078). Expression of CD8 was enhanced at the same time in $V\beta8^+$, $CD8^+$ T cells (Newell KA, Ellenhorn JD, Bruce DS, Bluestone JA (1991) Proc Natl Acad Sci USA 88:1074-1078). These SEB-"primed" cells proliferated in response to IL2 or TCR stimulation (SEB and allogenic stimulations) more efficiently and with quicker kinetics than normal cells when tested within 24 hours post priming (MacDonald HR, Baschieri S, Lees RK (1991) Eur J Immunol 21:1963-1966; Newell KA, Ellenhorn JD, Bruce DS, Bluestone JA (1991) Proc Natl Acad Sci USA 88:1074-1078). The level of activation as measured by IL-2R expression and

proliferation, depended on the dose of SEB injected, but expansion of Vβ8⁺ T cell proportion was usually less than 300 % and the reduction hardly reached below 50 % of normal level even at high doses (~800 μg) (unpublished results). γIFN was induced *in vivo* and detected in the serum of SEB-primed mice 10 hours after injection (Newell KA, Ellenhorn JD, Bruce DS, Bluestone JA (1991) Proc Natl Acad Sci USA 88:1074-1078). These cells activated *in vivo* were suggested to contribute to the prevention of outgrowth of a malignant tumor which was inoculated with SEB concomitantly (Newell KA, Ellenhorn JD, Bruce DS, Bluestone JA (1991) Proc Natl Acad Sci USA 88:1074-1078). By day 4 after SEB injection spleen cells lost most of the responsiveness (~80 %) to SEB despite the fact that spleen had similar or more number of Vβ8⁺ T cells than normal mice (MacDonald HR, Baschieri S, Lees RK (1991) Eur J Immunol 21:1963-1966; Newell KA, Ellenhorn JD, Bruce DS, Bluestone JA (1991) Proc Natl Acad Sci USA 88:1074-1078).

The administration of bacterial superantigens also induces specific T cell anergy *in vivo*. Mice injected i.p. or i.v. with SEB did not specifically respond when spleen or LN cells were studied 7 days after SEB challenge (Kawabe Y, Ochi A (1991) Nature 349:245-248; MacDonald HR, Baschieri S, Lees RK (1991) Eur J Immunol 21:1963-1966; and Rellahan BL, Jones LA, Kruisbeek AM, Fry AM, Matis LA (1990) J Exp Med 172:1091-1100). Similar clonal anergy was also induced in SEA-injected mice (MacDonald HR, Baschieri S, Lees RK (1991) Eur J Immunol 21:1963-1966). Anergy was measured in both proliferation and IL-2 production assays. The degree of anergy depended on the dose of SEB and became evident when the SEB dose was above 1 μg / mouse. Results were equivalent even when mice were challenged with SEB emulsified in complete Freund's adjuvant (Rellahan BL, Jones LA, Kruisbeek AM, Fry AM, Matis LA (1990) J Exp Med 172:1091-1100). A detailed study of T cells in SEB-primed spleen demonstrated that

only CD4$^+$ T cells and not CD8$^+$ were unresponsive (Kawabe Y, Ochi A (1991) Nature 349:245-248). Similar data which showed selective anergy of CD4$^+$ T cells were reported in a study of Mls-1^a mice expressing a transgenic Vβ8.1 beta chain (Blackman MA, Gerhard-Burgert H, Woodland DL, Palmer E, Kappler JW, Marrack P (1990) Nature 345:540-542). Although, these anergic Vβ8$^+$ T cells were able to express IL-2R, they failed to proliferate in response to exogenous IL-2 (Rellahan BL, Jones LA, Kruisbeek AM, Fry AM, Matis LA (1990) J Exp Med 172:1091-1100; and Rammensee H, Kroschewski R, Frangoulis B (1989) Nature 339:541-54439,41). Therefore, *in vivo* induced clonal anergy of T cells by superantigens appears to be different from that induced in Th cell clones *in vitro* in response to IL2. Recently, Blackman reported altered antigen signaling in anergic T cells of T cell receptor β-chain transgenic mice (Blackman MA, Finkel TH, Kappler J, Cambier J, Marrack P (1991) Proc. Natl. Acad. Sci. USA 88:6682-6686). These anergic T cells were defective in calcium mobilization and IL-2R was not membrane induced following TCR ligation.

The results may indicate that the defect of signaling pathway may be caused by phospholipase C-γ which plays important role in intra-cellular Ca^{++} mobilization. The present inventor obtained similar results in SEB-anergized Vβ8$^+$ T cells. Hence, it is proposed that there exist defects in trans-signaling cascades in TCR mediated pathways in SEB-anergic T cells. In any case, the results suggest that *in vivo* normal anergic T cells are hampered in their response primarily by an abnormality in a trans-signalling mechanism.

E. Failure to induce tolerance by TSST-1.

As previously discussed bacterial superantigens possess enormous potential to bias the functions as well as to alter the number of reactive Vβ TCR expressing T cells *in*

vivo. The activation, activation-induced PCD and anergy seem to represent *in vivo* effects to T cells in SEA- or SEB-treated mice. Because PCD and anergy have been recognized as major mechanisms involved in the development of central or thymic T cell tolerance, it was proposed that SE's induce tolerance in specific TCR Vβ positive T cells *in vivo*. In order to ask if this course of events is particular to some SEs, or is a general phenomena of superantigens, TSST-1, the least homologous member of SE family, was studied with regard to PCD and anergy *in vivo*.

Figure 4 shows the results of a DNA assay of spleen T cells of TSST-1 or SEB-treated Balb/c mice. Balb/c mice were injected i.p. with 50 µg of SEB or TSST-1 3 days before DNA assay by the methods as described previously (Kawabe Y, Ochi A (1991) Nature 349:245-248). In Figure 4 a. is the PBS-injected control mice, b. SEB-injected mice, and c. TSST-1-injected mice. It is clear that spleen T cells of both TSST-1 and SEB-treated mice contained cells programmed to die whereas the control spleen T cells (PBS-treated) did not have such cells. Spleen sizes of TSST-1 or SEB-primed mice were about 1.5 times larger than that of control and many blastic T cells were observed by microscopic assay (data not shown).

The results confirmed previous data of transient Vβ8 T cell expansion and death in SEB-treated mice (Kawabe Y, Ochi A (1991) Nature 349:245-248). It was also suggested that TSST-1 stimulated and induced PCD in T cells in injected mice. Unfortunately, the specific antibodies to Vβ15 and Vβ16 products were not available and assays for those TSST-1 reactive Vβ TCRs expressing cells in spleen were not determined. However, as the expansion of Vβ8 or Vβ6 T cells in TSST-1 primed spleen T cells was not observed this supports the fact that the T cell blast induction observed on day 3 is not a nonspecific effect like that of plant T cell mitogens. The spleen cells of

12 days TSST or SEB-primed mice were then examined (Table 3). Pre-injection of 50 µg of SEB reduced the response *in vitro* after 12 days but the pre-injection of TSST-1 at various doses did not reduce *in vitro* response to TSST-1. Thus, the data indicated that SEB and TSST-1 are different concerning anergy induction to specific T cells. The data presented the first evidence that SEs are heterogeneous in regard to T cell tolerance *in vivo*.

The presented data distinguish TSST-1 from SEA and SEB with respect to its *in vivo* effect on T cells, that is, SEA and SEB induce tolerance in treated mice whereas injection of TSST-1 does not result in tolerance. Therefore, previous observations which relate superantigens to the suppression of reactive Vβ TCR T cells seem difficult to generalize to all bacterial superantigens (it may be true for viral superantigens). TSST-1 has various differences from other members of SE (summarized in Table 4). It has a small amino acid sequence homology and lacks middle region located disulfide loops, unlike other SEs. This molecule binds to a site of class II MHC different from SEA or SEB and also does not bind to I-E molecules unlike SEA or SEB. In addition to these molecular differences, the *in vivo* sequelae of TSST-1 is development of the TSS rather than food poisoning which is highly associated with SEA and SEB (Bergdoll MS: Enterotoxins, in Easmon CSF, Adlam C (eds) (1983) Staphylococci and staphylococcal infections, vol.2,chapter 16: The organism *in vivo* and *in vitro*. Academic Press, New York pp559-598 and Marrack P, Kappler J (1990) Science 248:705-711). Since lymphokine (eg.TNF) production by SE-stimulated T cells is part of the pathogenesis for TSS (Miethke T, Wahl C, Heeg K, Echtenacher B, Krammer PH, Wagner H (1992) J Exp Med 1992;175:91-98), it is possible that the failure to induce anergy by TSST-1 may prolong the lymphokine over production by reactive Vβ TCR positive T cells and elicit

TSS.

Previously, the induction mechanism of protein antigen-specific unresponsiveness of T helper cells was thought to be the result of occupancy of the TCR in the absence of costimulatory signals (Mueller DL, Jenkins MK, Schwartz RH (1989) Ann Rev Immunol 7:445-480 and Schwartz RH (1990) Science 248:1349-1356). Therefore, TSST-1 seems to satisfy the requirement of costimulatory signals whereas SEA and SEB do not involve costimulatory signals to stimulate T cells. It is an intriguing question how TSST-1 does the task of providing costimulatory signals to T cells. One possibility may be that TSST-1 has an affinity to a costimulatory receptor on the T cells or could be complexed with costimulatory "substrates" on the APC and stimulate T cells in complexed form with class II MHC-costimulatory substrates. T cells thereby stimulated by both TCR and costimulatory molecules will avoid anergy after all. Alternatively, TSST-1 may induce costimulatory substrates on APC. This seems to be possible because APCs are able to be activated by SEs. Since TSST-1 apparently binds to class II MHC in a different fashion to SEA and SEB, the scale or the nature of signals triggered in APC may be different between TSST-1 and other SEs. If the TSST-1, but not SEA and SEB, efficiently induces co-stimulatory substrates on APC, only TSST-1 will not cause anergy in reactive T cells.

The possibilities discussed above have been based on the assumption that conventional protein antigens and SEs induce T cell anergy in the same way. However, unresponsiveness to superantigens may arise by different mechanisms because SE's, represented by SEB, SEA, and TSST-1 in this report, have a number of striking differences from classical protein antigens. These are: 1) unique binding to class II MHC outside of the antigen groove; 2) non-requirement of processing by APC to

stimulate T cells; 3) strict specificity for TCR β chain V region; and 4) no requirement for the CD8 co-receptor molecule to stimulate CD8 T cells. In addition to these possibilities, there is a fifth, controversial, suggestion that there are different trans-signaling pathways used by superantigens (O'Rourke AM, Mescher MF, Webb SR (1990) Science 249:171-174; Chatila T, Wood N, Parsonnet J, Geha RS (1988) J Immunol 140:1250-1255; and Liu H, Lampe MA, Lregui MV, Cantor H (1991) Proc Natl Acad Sci USA 88:8705-8709). Because of the uniqueness of superantigen mediated T cell stimulation, it may be that induction of anergy via superantigens uses different pathways than induction of anergy by classical antigens.

The differences between TSST-1 and SEB in terms of induction of tolerance suggest that all superantigens may not generally be useful in immunotherapy. Each superantigen should be studied *in vivo* in respect to T cell tolerance induction.

Example 3

T cell tolerance to antigens presented in extrathymus has been examined using transgenic mice which carry allogenic histocompatibility antigen or allo-antigen transgenes (Lo, D., L. C. Burkly, G. Widera, C. Cowing, R. A. Flavell, R. D. Palmiter, and R. L. Brinster. 1988. Cell 53: 159; Allison, J., I. L. Campbell, G. Morahan, T. E. Mandel, L. C. Harrison, and J. F. A. P. Miller. 1988 Nature 333: 529; Burkly, L. C., D. Lo, O. Kanagawa, R. L. Brinster, and R. A. Flavell. 1989 Nature 342: 564; Murphy, K. M., C. T. Weaver, M. Elish, P. M. Allen, and D. Y. Loh. 1989. Proc. Natl. Acad. Sci. USA 86: 10034. Blackman, M. R., H. Gerhard-Burgert, D. L. Woodland, E. Palmer, J. W. Kappler, and P. Marrack. 1990 Nature 345: 540; and Wieties, K., R. E. Hammer, S. Jones-Youngblood, and J. Forman. 1990. Proc. Natl. Acad. Sci. USA 87: 6604) or adult mice tolerized against alloantigens, viral superantigens, and

bacterial superantigens (Martin, R. D., and R. G. Miller. 1989 J. Exp. Med. 170: 679; Rammensee, H., R. Kroschewski, and B. Frangoulis. 1989 Nature 339: 541; Qin, S., S. Cobbold, R. Benjamin, and H. Waldmann. 1989 J. Exp. Med. 169: 779; Kawabe, Y., and A. Ochi. 1990 J. Exp. Med. 172: 1065; Rellahan, B. L., L. A. Jones, A. M. Kruisbeek, A. M. Fry, and L. A. Matis. 1990 J. Exp. Med. 172: 1091; Webb, S., C. Morris, and J. Sprent. 1990 Cell 63: 1249; Jones, L.A., L. T. Chin, D. L. Longo, and A. M. Kruisbeek. 1990 Science 250: 1726; Kawabe, Y., and A. Ochi. 1991. Nature 349: 245; and Rocha, B., and H. von Boehmer. 1991. Science 251: 1224). These experiments demonstrated that tolerance of T cells in the periphery may be achieved by clonal deletion of T cells by activation induced programmed cell death (PCD) as well as functional unresponsiveness (anergy) of mature T cells. PCD appears to be a general phenomenon in the studies of various cells (Appleby, D. W., and S. P. Modak. 1977 Proc. Natl. Acad. Sci. USA 74: 5579;Oppenheim, R. W., D. Prevette, M. Tytell, and S. Homma. 1990 Dev. Biol. 138: 104; Ucker, D. S., J. D. Ashwell, and G. Nickas. 1989. J. Immunol. 143: 3461; Zacharchuk, C. M., M. Mercep, P. K. Chakraborti, S. S. Simons, Jr., and J. D. Ashwell. 1990. J. Immunol. 145: 4037; Ellis, H. M., and H. R. Horvitz. 1986 Cell 44: 817; and Schwartz, L. M., L. Kosz, and B. K. Kay. 1990 Proc. Natl. Acad. Sci. USA 87: 6594). In contrast, anergy seems to be exclusive for lymphocytes. Induction of specific T cell tolerance in Staphylococcal enterotoxin B (SEB)-primed mice has previously been reported (Kawabe, Y., and A. Ochi. 1990 J. Exp. Med. 172: 1065; Rellahan, B. L., L. A. Jones, A. M. Kruisbeek, A. M. Fry, and L. A. Matis. 1990 J. Exp. Med. 172: 1091; Webb, S., C. Morris, and J. Sprent. 1990 Cell 63: 1249; and Kawabe, Y., and A. Ochi. 1991. Nature 349: 245). Tolerance was evident when spleen cells of SEB-primed mice were examined for the *in vitro* proliferative response to SEB after a week. SEB-specific IL-2 production was also severely depressed in tolerant

mouse spleens. The results showed SEB-specific unresponsiveness occurred more severely in $CD4^+$, $V\beta8^+$ T cells rather than $CD8^+$, $V\beta8^+$ T cells. In this investigation the kinetics of spleen cell unresponsiveness of SEB tolerant thymectomized Balb/c mice is examined. In these mice, suppressed response continued more than 2 months but recovered by 4 months after tolerance induction. The response efficiency of purified $CD4^+$, $V\beta8^+$ T cells after 4 months was the same as that of the control mice. The study of the surface phenotype showed that these "awakened" cells express LFA-1, Ly-24, and CD45R at a similar level to those of control cells but the homing receptor was slightly decreased. Finally, an experiment was performed which suggested that it is unlikely that SEB-reactive $CD4^+$, $V\beta8^+$ T cells of 4 months SEB-primed thymectomized mice originate from $CD4^+$, $V\beta8^+$ T cells that were not rendered anergic by *in vivo* administration of SEB. The data demonstrate that the anergic state of $CD4^+$, $V\beta8^+$ T cells is reversible *in vivo*. The relationships of these cells to memory T cells is not clear.

The following materials and methods were used in the investigations described in this example:

Mice.

Balb/cbyJ and thymectomized Balb/cbyJ mice were obtained from Taconic (Germantown, NY). C57BL/6 (Thy1.2) and its Thy1.1 congenic strain were obtained from The Jackson Laboratory (Bar Harbor, ME).

Antibodies, mitogens and lymphokines.

B cell hybridoma lines producing rat antibodies directed against murine Thy1.1 (T1107e2), Thy-1.2 (HO-13-4), CD4 (GK1.5), CD8 (3.155), CD45R (MB23G), LFA-1 (M17/4.2), and homing receptor (Mel-14) (22-24) were purchased from the American Type Culture Collection (ATCC), (Rockville, MD). Monoclonal rat IgG antibody directed against Ly-24 (PgP-1)

was obtained from Dr. R. Hyman (Trowbridge, I. S., J. Lesley, R. Shulte, R. Hyman, and J. Trotter. 1982. Biochemical characterization and cellular distribution of a polymorphic, murine cell-surface glucoprotein expressed on lymphoid cells. Immunogenetics 15: 229). Vβ8.1,2-specific monoclonal antibody, KJ-16 (Haskins, K., C. Hannum, J. White, N. Roehm, R. Kubo, J. W. Kappler, and P. Marrack. 1984. The major histocompatibility complex-restricted antigen receptor on T cells. VI. An antibody to a receptor allotype. J. Exp. Med. 160: 452) was provided from Dr. P. Marrack. Vβ8.1,2,3-specific monoclonal antibody, F23.1 (Staerz, U. D., H-G. Rammensee, D. J. Benedetto, and J. M. Bevan. 1985. Characterization of a murine monoclonal antibody specific for an allotype determinant on T cell antigen receptor. J. Immunol. 134: 3994) was provided from Dr. M. J. Bevan. This antibody and Thy1.1, Thy1.2-specific antibodies were purified and biotinylated. Vβ6-specific monoclonal antibody, RR4-7 (Kanagawa, O. 1989. *In vivo* T cell tumor therapy with monoclonal antibody directed to the Vβ chain of T cell antigen receptor. J. Exp. Med. 170: 1513) was provided from Dr. O. Kanagawa. Phycoerythrin (PE)-anti-CD4 antibody and PE-avidin were purchased from Becton Dickinson & Co. (B-D), (Mountain View, CA). FITC-goat anti-rat IgG (absorbed with mouse immunoglobulin) was purchased from Tago Inc. (Burlingame, CA). SEB was purchased from Sigma Chemical Co. (St. Louis, MO). SEA was purchased from Toxin Tec., Inc. (Madison WI).

Preparation of cells.

$CD4^+$, $V\beta8^+$ T cells.

Thymectomized Balb/cByJ mice were given intraperitoneal injections of 100 μg SEB in 0.2 ml PBS to induce SEB-specific tolerance. In control mice 0.2 ml of PBS was injected. Four months later, spleen single cell suspensions were treated with Tris buffered 0.16 M Ammonium chloride to lyse the red blood cells (RBC). In

order to deplete $CD8^+$ T cells spleen cells were treated with anti-CD8 B cell hybridoma supernatant and 1:12 diluted guinea pig serum (Calbiochem, San Diego, CA) as the complement source for 1 hr at 37°C. After washing twice cells were incubated for 30 min on ice with biotinylated F23.1 antibody at 10 μg of antibody per $5x10^7$ cells in 1ml. Cells were washed and mixed with Streptavidin-magnetic beads (Dynabeads M280, DYNAL A.S., Oslo, Norway) at 10 $\mu l/10^5$ cells for 30 min at room temperature (r.t.). The magnetically coated cells were isolated by a magnetic field generated by MACS, (Miltenyi Biotec GmbH., Meitzfeld, West Germany). Purification for positively coated cells was performed three times.

Spleen non-T cells (non T cells).

Balb/c spleen cells were treated with anti-Thy1.2 B cell hybridoma supernatant at 10^7 cells per 30 ml for 1 hr at r.t.. Cells were washed and resuspended to 1:12 diluted guinea pig serum at 10^7 cells/ml and incubated for 1 hr at 37°C. Cells were washed and irradiated with 3000R to stop cell growth.

<u>Proliferation assays.</u>

Unfractionated spleen cells were *in vitro* stimulated with SEB and SEA in 96 multi-well U bottom tissue culture plates at a reciprocal number ($1 - 8 \times 10^4$) of cells/100 μl in each well. In an experiment purified 5×10^4 of $CD4^+$, $V\beta8^+$ T cells and 2×10^5 of non-T cells were *in vitro* stimulated with reciprocal dose (1-100 μg/ml) of SEB in 96 multi-well U bottom tissue culture plates. After 40 hrs of culture, cells were pulsed with 1 μCi/well of [^{3}H]-Thymidine (Amersham, Buckinghamshire, England). Cells were collected 8 hrs later onto glass fiber filter mats by a microsample harvester (Skatron Inc.). Radioactivity was determined in a liquid scintillation β counter. Data are indicated as arithmetic means of quadruplicate samples.

<u>Flow microfluorometry.</u>

RBC depleted spleen cells were treated with anti-CD8 antibody and 1:12 diluted guinea pig serum as mentioned above. $CD8^-$ spleen cells were incubated for 1 hr on ice with various rat monoclonal antibody producing B cell hybridoma supernatants at 10^6 cells per 0.5 ml supernatant. They were then washed and stained with FITC goat anti-rat IgG. After intensive washings cells were incubated with biotinylated F23.1 at 5 $\mu g/10^6$ cells for 1 hr on ice then stained with PE-streptavidin. To determine the proportion of $V\beta8^+$ or $V\beta6^+$ T cells in the total CD4 or CD8 populations, RBC depleted spleen cells were incubated with biotinylated F23.1, washed and incubated with PE-streptavidin. Cells were then incubated with either anti-CD4 or anti-CD8 rat antibody followed by FITC-goat anti-rat IgG. $V\beta6^+$ cells were assayed using RR4-7, and FITC-goat anti-rat IgG. The cells were then treated with PE (red)-anti-CD4 antibody or biotinylated anti-CD8 for 30 min. To determine CD8 expression, cells were washed and further incubated with PE-avidin for another 30 min. The percentage of $CD4^+$ T cells expressing Vβ8 and Vβ6 was calculated by dividing the number of cells stained with both anti-Vβ8 and anti-CD4 antibodies by the total number of cells stained with anti-CD4 antibody. The percentage of $CD8^+$ T cells expressing Vβ8 and Vβ6 was calculated in the same manner. Each analysis was done on $2x10^4$ cells.

<u>Preparation and analysis of spleen cell chimera.</u>

C57BL/6 (Thy1.2) mice were primed i.p. with 100 μg SEB in 0.2 ml PBS. One week after injection spleen single cell suspension was RBC depleted. These prepared SEB-anergic spleen cells were mixed with 5 times the number of goat-anti-rat Igs magnetic beads (Dynabeads M450) saturated with CD4 or CD8-specific rat monoclonal antibodies. After 30 min of incubation at r.t. $CD4^+$ or $CD8^+$ T cells were positively purified by MACS. These cells (2.5 X 10^7) and similarly prepared CD4 or CD8 spleen T cells (2.5 X 10^7) of

normal Thy1.1 congenic C57BL/6 mice were mixed and injected intraperitoneally to each C57BL/6 Thy1.1 mouse that was 600R irradiated one week before. Mice were monitored for the proportion of Thy1.1 or Thy1.2 cells in Vβ8.1,2⁺ T cells as well as the response to SEA and SEB of Thy1.1 or Thy1.2 cells.

a) Proliferative response to SEB or SEA.
Spleen cells were treated with anti-Thy1.1 or anti-Thy1.2 antibody and complement to deplete either phenotype cells. After treatment, cells (10^5/well) were stimulated with SEB (10 μg/ml) or SEA (2.5 μg/ml) *in vitro* for proliferation assay.

b) Proportion of Thy1.1 or Thy1.2 cells in $V\beta8^+$ T cells.
Spleen cells of Thy1 chimeric mice were incubated with anti-Vβ8.1,2 (Rat Ig) (KJ16). Cells were then stained with FITC-goat anti-Rat IgG. After washing three times, the cells were incubated with biotinylated anti-Thy1.1 or anti-Thy1.2 which was followed by treatment with PE-streptavidin. The FITC-stained $V\beta8^+$ T cell population was determined by software gating and PE fluorescence, presented by a single parameter histogram.

<u>A. SEB-specific response of SEB-primed thymectomized mouse spleen after 10 days.</u>

Thymectomized Balb/cByJ mice were primed with 100 μg SEB to induce SEB-specific T cell tolerance. After 10 days, spleen cells were studied for their proliferative response to SEB (Fig. 5). Figure 5 shows SEB-specific spleen cell response on day 10. Varied doses of spleen cells (horizontal axis) were cultured in the presence of 10 μg/ml SEB (circles) or 2.5 μg/ml SEA (triangles) 10 days after SEB-priming. Open circles and a triangle: control spleen cells; black circles and a triangle: SEB-primed spleen cells. Compared to the cells from PBS-primed mice

(open circles) spleen cells from mice previously primed with 100 µg SEB showed reduced thymidine incorporation in response to SEB (black circles) at all cell doses. The response to SEA was comparable in both (open and black triangles). IL-2 production in response to *in vitro* SEB stimulation was also suppressed in the same spleen cells. Thus, SEB-specific peripheral tolerance after 10 days in SEB-primed thymectomized mice was confirmed.

B. SEB-specific response of SEB-primed thymectomized mouse spleen after 2 months.

Spleen cells were assayed *in vitro* for SEB-specific proliferative responses 2 months after SEB-priming (Fig. 6). Figure 6 shows SEB-specific spleen cell response on 2 months. Varied doses of spleen cells (horizontal axis) were cultured in the presence of 10 µg/ml SEB (circles) or 2.5 µg/ml SEA (triangles) 2 months after SEB-priming. Open circles and a triangle: control spleen cells; black circles and a triangle: SEB-primed spleen cells.
The response was higher than after 10 days post SEB-priming as shown in Figure 5. However, the response was still below that of control spleen cells. The response to SEA was again comparable with both types of spleen cells (black and open triangles). Thus, mice were apparently recovering from tolerance two months after SEB challenge.

C. SEB-specific response of SEB-primed thymectomized mouse spleen after 4 months.

When SEB-primed spleen cells were examined 4 months later they responded efficiently to SEB and the cell dose dependent response curve was comparable to that of control spleen cells (Fig. 7a).

Figure 7 shows SEB-specific spleen cell response on 4 months (a.) Varied doses of spleen cells (horizontal axis)

were cultured in the presence of 10 μg/ml SEB (circles) or 2.5 μg/ml SEA (triangles) 4 months after SEB-priming. Open circles and a triangle: control spleen cells; black circles and a triangle: SEB-primed spleen cells. (b.) 5 x 10^4 of $CD4^+$, $V\beta8^+$ T cells were cultured with 2 x 10^5 of irradiated non-T cells in the presence of varied doses of SEB (horizontal axis). Open circles: control $CD4^+$, $V\beta8^+$ T cells; black circles: SEB-primed $CD4^+$, $V\beta8^+$ T cells.

IL-2 production of SEB-stimulated spleen culture also recovered to control level. In order to examine the response recovery in more detail, $CD4^+$, $V\beta8^+$ T cells were purified from spleen and their response to SEB determined. The data shown in Figure 7b indicate that $CD4^+$, $V\beta8^+$ T cells of SEB-primed and control mice possess identical responsiveness. Therefore, anergic T cells regained their proliferative response to SEB 4 months after *in vivo* induction of unresponsiveness.

D.Phenotype study.

In order to examine if any phenotypic change accompanies this awakening from unresponsiveness, the surface molecules of these $CD4^+$, $V\beta8^+$ T cells was examined using various monoclonal antibodies (Table 5). The results indicated that these cells expressed Ly-24, CD45, and LFA-1 at similar levels of unprimed control $CD4^+$, $V\beta8^+$ T cells but Mel-14 was slightly decreased. This phenotype was different from that at days 7 and 10, which expresses high Ly-24 and LFA-1, (Table 5), and was also different from the reported phenotype of memory T cells, which expresses high Ly-24, LFA-1 but low CD45R (Budd, R. C., J-C. Cerottini, C. Horvath, C. Bron, T. Pedrazzini, R. C. Howe, and H. R. MacDonald. 1987 J. Immunol. 138: 3120; Butterfield, K., C. G. Fathman, and R. C. Budd. 1989. J. Exp. Med. 169: 1461; Sanders, M. E., M. W. Makgoba, S. O. Sharrow, D. Stephany, T. A. Springer, H. A. Young, and S. Shaw. 1988 J. Immunol. 140: 1401; and Springer, T. A.

1990. Nature 346: 425). Expression of CD4 and CD3 was also identical to normal cells in both anergic and recovered T cells. Therefore, recovered $CD4^+$, $V\beta8^+$ T cells appear to express a phenotype only subtly different from unprimed cells.

E. Kinetics of $V\beta8^+$ T cells in SEB-tolerant thymectomized mice.

Previous reports have documented that SEB-priming causes the reduction of $V\beta8^+$ T cells by activation induced PCD as well as induction of unresponsiveness of peripheral cells (Kawabe, Y., and A. Ochi. 1991 Nature 349: 245 and Rocha, B., and H. von Boehmer. 1991 Science 251: 1224). This reduction was evident in both euthymic and athymic mice and was exclusive for $CD4^+$, $V\beta8^+$ T cells. It was queried whether the recovery of the SEB-specific response mirrored a proportional change of $V\beta8^+$ T cells in the periphery. Therefore, the proportion of $V\beta8^+$ cells in the spleen was determined (Table 6). Ten days after SEB injection, one third of the $V\beta8^+$ population was deleted in the CD4 but not in the CD8 subset. The $V\beta6^+$ cells which do not respond to SEB were not changed. In summary, the percentage of these cells on day 10 was maintained even after 4 months and there was no increase in total number of $CD4^+$, $V\beta8^+$ spleen T cells.

F. Is the recovery of $CD4^+$, $V\beta8^+$ T cells because of the expansion of non-anergized $CD4^+$, $V\beta8^+$ T cells *in vivo*

The above-mentioned results demonstrated that response of $CD4^+$, $V\beta8^+$ T cells recovers *in vivo* from SEB-induced anergy within 4 months. They also demonstrate that the proportions of $V\beta8^+$ or $V\beta6^+$ cells are stable during the period of recovery. These data do not absolutely exclude the possibility that the responding cells 4 months after SEB-injection were actually derived from non-anergized

$CD4^+$, $V\beta8^+$ T cells which existed in small numbers in SEB-anergic spleens. However, this possibility, to be justified, must satisfy three major conditions: 1) non-anergic $CD4^+$, $V\beta8^+$ T cells that exist in small population (~10 %) in SEB-tolerized spleen $CD4^+$, $V\beta8^+$ T cells (unpublished data) increase their number *in vivo*; 2) anergic $CD4^+$, $V\beta8^+$ T cells which occupy the rest (~90 %) must die; and 3) an increase of non-anergic cells must be balanced with the decrease of anergic $CD4^+$, $V\beta8^+$ cells, retaining the proportion of total $CD4^+$, $V\beta8^+$ cells unchanged. In order to further the investigation of whether these conditions are realistic or not, an experiment was performed in which the same number of SEB-anergized (C57BL/6, Thy1.2) and control non-anergic (C57BL/6, Thy1.1) spleen CD4 and CD8 cells were parked together in 600R irradiated C57BL/6, Thy1.1 host. These spleen cell chimeras were monitored for both the SEB and SEA responses as well as the proportion of Thy1.1 and Thy1.2 cells in $V\beta8^+$ T cells until the response of SEB-anergized Thy1.2 cells recovered. In this spleen cell chimera, ~60 % of $CD4^+$, $V\beta8^+$ T cells are non-anergic and most of them are $Thy1.1^+$ phenotype. Owing to the large proportion of Thy1 marked non-anergic T cells, if non-anergic $V\beta8^+$ T cells reconstitute the animal and anergic $V\beta8^+$ T cells die *in vivo*, an increased proportion of Thy1.1, $V\beta8^+$ T cells will easily be seen. However, a significant change was not seen in the ratio between Thy1.1 and Thy1.2, $V\beta8^+$ T cells for 8 weeks at which time Thy1.2 T cells responded to SEB (Figure 8).

Figure 8 shows the kinetics of SEB-response recovery and $V\beta8^+$ cell proportion in non-anergic (Thy1.1) and anergic (Thy1.2) chimeric mice. SEB-induced proliferative response of spleen cells treated with anti-Thy1.2 or anti-Thy1.1 and complement were denoted Thy1.1 (open columns) and Thy1.2 (black columns) respectively. Oblique lined narrow columns beside each Thy1 columns indicate the response of

each cells to SEA. ^{3}H-Thymidine incorporation by stimulated cells were denoted as CPM. Data indicated the average and standard deviations of quadruplicated samples. Diagrams in right side of each Thy1 columns are the result of flow microfluorometry studies on the proportions of Thy1.1 or Thy1.2 cells in Vβ8$^+$ T cells as indicated in columns. Data were presented as two-dimensional counter maps. Vertical axis indicate number of cells and horizontal axis indicate fluorescence in log scale. The FITC-stained (Vβ8$^+$) Thy1.1$^+$ or Thy1.2$^+$ (PE-stained), T cell population was determined by software gating and PE fluorescence were presented by a single parameter histogram. Onset: SEB-anergic spleen cells (Thy1.2) and normal spleen cells (Thy1.1) are mixed at 1:1 and assays were done immediately. 4W and 8W: data obtained four and eight weeks after the administration of the purified CD4 and CD8 T cells.

The data indicate that *in vivo* parked non-anergic Vβ8$^+$ T cells do not increase in proportion. There may be an argument that the failure to detect the proliferation of non-anergic Vβ8$^+$ T cells in irradiated hosts is because of the lack of SEB which is retained in very small amounts in the SEB-primed animals. However, this assumption will not satisfy the second condition that anergic T cells must die *in vivo*. The presented data also denied the possibility that an unknown factor from anergic T cells (if it exists) promotes the growth of non-anergic Vβ positive T cells. In summary, the data was not in favor of the possibility that non-anergic T cell proliferated to replace the anergic T cells *in vivo*. Therefore, the other possibilities, outlined above also appeared to be unlikely. In addition to these results it was recognized that there was no sign of apoptosis in purified anergic CD4$^+$, Vβ8$^+$ T cells studied by a DNA degradation assay. Thus, in summary, the possibility that anergic T cells die and non-anergic T cells repopulate the periphery to

account for the reversal of anergy to SEB appears unlikely.

The above study has investigated the *in vivo* fate of anergized CD4$^+$, Vβ8$^+$ T cells. The study indicated that unresponsiveness of CD4$^+$, Vβ8$^+$ T cells is reversible and after four months cells regain a proliferative response comparable to that of control mice. These "awakened" cells seemed to have a homologous phenotype to that of resting CD4$^+$, Vβ8$^+$ T cells, although a homing receptor was slightly reduced. The proportion of CD4$^+$, Vβ8$^+$ T cells remained low after activation induced deletion took place within the first week (Kawabe, Y., and A. Ochi. 1991 Nature 349: 245). These results indicate that anergy of T cells is reversible *in vivo*.

The results also showed that SEB-specific peripheral tolerance lasts for a long period of time. This did not require boosting of SEB, as a single injection was sufficient. Interestingly, CD4$^+$, Vβ8$^+$ T cell tolerance in SEB-primed euthymic mice was also long lasting. This seems not to be unique for SEB but is a common phenomena in peripheral T cell tolerance seen in various antigen systems (Rammensee, H., R. Kroschewski, and B. Frangoulis. 1989 Nature 339: 541; Roser, B. J. 1989 Immunological Reviews 107: 179; Macphail, S., O. Stutman. 1989. J. Immunol. 143: 1795; Heeg, K., and H. Wagner. 1990. J. Exp. Med. 172: 719). To interpret this persistence of T cell unresponsiveness *in vivo* an underlying active control mechanism may be assumed. Although no evident mechanism has been reported yet it is possible that antigen is retained in a form that induces T cell unresponsiveness by antigen presenting cells for a long time. It is also possible that the molecular change in anergic T cells is so stable that newly synthesized intracellular proteins can not correct it. Alternatively, a certain cellular mechanism may keep T cells in unresponsiveness. Although

such a "suppressor cell" has not clearly been demonstrated to exist, a report has shown that γ/δ T cells break oral tolerance (Fujihashi, K., H. Kiyono, W. K. Aicher, D. R. Green, B. Singh, J. H. Eldrige, and J. R. McGhee. 1989. J. Immunol. 143: 3415) and may suggest the existence of a suppressor cellular regulatory system.

Of interest concerning the period for recovery is that T cells parked in irradiated host or nude mice apparently shortens anergy. Similar "quicker awakening" was also observed when SEB-anergic Balb/c ($H-2^d$) spleen cells were parked in severe combined immunodeficient mice ($H-2^d$) (data not shown). It took 1-2 months in these hosts while it took more than 3 months in SEB injected mice. This may suggest that a small amount of SEB-deposit in spleen prolongs the anergy as was suggested by Ramsdell (Ramsdell, F., and B. J. Fowlkes. 1992 Science 257: 1130) or a possible difference in microenvironments trigger signals in anergic cells to accelerate the response recovery. Difference in the term of recovery in different environment may suggest the possibility that we can artificially control the anergy of T cells. Although, the awakened $CD4^+$, $V\beta8^+$ T cells have a phenotype similar to that of control unprimed $CD4^+$, $V\beta8^+$ T cells and respond to SEB with comparable efficiency, they need not be actively functionally identical to unprimed cells. For example, the unfractionated "awakened" spleen cells and control spleen cells responded identically to SEB despite the fact that proportion of $CD4^+$, $V\beta8^+$ T cells was reduced to approximately 60 % in the former cells. This may be because of the low accuracy of proliferation assays to measure the population size or the awakened $CD4^+$, $V\beta8^+$ T cells may acquire helper function to induce stronger responses in another SEB-responsive population, $CD8^+$, $V\beta8^+$ T cells. In any case, the study of awakened T cells should be extended to other functional parameters such as production of various lymphokines and determination of

other surface markers such as vary late antigens (Yokoyama, W. M., S. R. Maxfield, and E. M. Shevach. 1989 Immunological Reviews 109: 153.) to obtain more strict evaluation.

Interestingly, the results discussed above seem to indicate that SEB-priming does not result in clonal expansion or memory of $CD4^+$, $V\beta8^+$ T cells. This is opposite to the current ideas of antigen induced secondary T cell responses (Burnet, F. M. 1959. The clonal selection theory of acquired immunity. In The Abraham Flexner Lectures: 1958. Cambridge University Press, Cambridge and Cerottini, J. C., and H. R. Macdonald. 1989. The cellular basis of T cell memory. Annu. Rev. Immunol. 7: 77), where antigenic stimulation results in more efficient secondary responses and the scale of memory is proportional to the antigenicity. SEB-stimulation may not result in memory because the superantigen induced T cell stimulation is extremely high and may exceed the upper limit which can be processed as antigen-specific T cell memory. Thus, there may be a stimulation "window" to integrate the T cell memory available for the secondary response, and the antigen determinants which have a stimulation level unfit for that window will not be stored as memory.

Example 4

T cell receptor (TCR)-mediated signal transduction in mouse spleen $V\beta8^+$ T cells anergized *in vivo* by intraperitoneal injection of the bacterial superantigen, Staphylococcal enterotoxin B (SEB) was examined as described in detail below. It was found that increases in tyrosine phosphorylation of cellular proteins, either in response to T cell-SEB-antigen presenting cell (APC) contact, or to cross-linking of TCR by specific monoclonal antibody are barely detectable in anergic T cells. Cross-

linking of TCR fails to generate inositol phosphates in anergic T cells. Anergic Vβ8$^+$ T cells possess normal amounts of CD3-ζ and the protein tyrosine kinase (PTK)-*fyn* which have been implicated in signal transduction in normal T cells. When the purified TCR complex was studied in *vitro* kinase assays, TCR stimulation failed to activate PTK-*fyn* in anergic Vβ8$^+$ T cells while the same stimulation dramatically induced TCR-associated PTK-*fyn* activity in normal T cells. Together with the observations that 1) stimulation of Vβ8$^+$ T cells with phorbol 12-myristate 13-acetate (PMA) and ionomycin induces normal proliferation and IL-2 production in these cells, and 2) anergic Vβ8$^+$ T cells possess normal amounts of functional tyrosine phosphatase activity, these observations indicate that anergy may be a consequence of failure to activate TCR-associated PTK(s).

Balb/c mice were primed with 100 µg of SEB by single intraperitoneal injection to induce anergy in peripheral Vβ8$^+$ T cells (Kawabe, Y., & Ochi, A. J. Exp. Med. 172, 1065-1070. (1990); Rellahan, B. L., Jones, L. A., Kruisbeek, A. M., Fry, A. M. & Matis, L. A. J. Exp. Med. 172, 1091-1100. (1990); and MacDonald, H. R., Baschieri, S. & Lees, R. K. Eur. J. Immunol. 21, 1963-1966. (1991)). Seven days after SEB administration both CD4$^+$ and CD8$^+$, Vβ8$^+$ spleen T cells responded poorly *in vitro* to SEB (<10 % of experimental value in IL-2 production assays). The unresponsiveness was not due to a loss of membrane TCRs because the Vβ8 TCR level as assayed by flow cytometry was similar to that of normal cells (Rellahan, B. L., Jones, L. A., Kruisbeek, A. M., Fry, A. M. & Matis, L. A. J. Exp. Med. 172, 1091-1100. (1990)). In SEB-tolerized spleen, Vβ8$^+$ cells comprise about 25 % of total T cells whereas normal spleen T cells contain approximately 30% Vβ cells (Kawabe, Y., & Ochi, A. Nature 349, 245-248. (1991)). On the other hand, Vβ6$^+$ T cells from spleen, which contain SEB-non-reactive cells, responded to the

minor lymphocyte stimulating (Mls) antigen-1[a] at a level equivalent to cells from mice not pre-treated with SEB. When SEB-anergized spleen T cells were stimulated with Vβ8-specific antibody, inositol phosphates (Ips) were not generated, while the same antibody stimulation generated Ips in control spleen T cells (Fig. 9A) suggesting the defect is at the level of the TCR activation. This assumption was further supported by the fact that co-stimulation with PMA plus ionomycin restored more than 80 % of IL-2 production in purified anergic Vβ8$^+$ T cells (Fig. 9B, C, D).

Using SEB-tolerized spleen T cells, cellular protein tyrosine (tyr) phosphorylation was examined in response to stimulation through the Vβ8-TCR by SEB or by Vβ8-specific antibodies (Fig. 10). When spleen T cells of PBS-treated control mice were stimulated with SEB in the presence of the fixed class II major histo-compatibility complex (MHC) antigen-positive B lymphoma, A20-2J (13), (Fig. 10A), or cross-linking of the TCR with anti-Vβ8 antibody (F23.1) (Staerz, U. D., Rammensee, H-G., Benedetto, D. J. & Bevan, J. M. J. Immunol. 134, 3994-4000. (1985)), (Fig. 10B), an increase in the amount of tyr-phosphorylation was readily detected even though the activation occurs in less than 30 % of the total cells. In contrast, when SEB-tolerized spleen T cells were similarly stimulated, an increase in the amount of tyr-phosphorylated proteins was low and mostly insignificant. Thus, the data indicate that TCR stimulation failed to induce a notable increase in tyr-phosphorylation in anergic Vβ8$^+$ T cells.

It is difficult to quantitate the difference in tyr-phosphorylation between anergic and control spleen T cells in the previous results, because Vβ8$^+$ T cells constitute only 30 % of the total cells. In order to examine in more detail the downstream tyr-phosphorylation events in TCR-stimulated anergic T cells, TCR was immuno-precipitated

and assayed for the tyr-phosphorylated components. Shown in Fig. 11A is the data by phosphor-tyrosine (ptyr)-specific western blots, where the bands at 21 K dalton (Kd) shows tyr-phosphorylated CD3-ζ (Samelson, L. E., Patel, M. D., Weissman, A. M., Harford, J. B. & Klausner, R. D. Cell 46, 1083-1090. (1986)). In control cells, the amount of tyr-phosphorylated CD3-ζ was increased (~3 times based on the values of arbitrary unit by densitometry analysis) when the TCR was stimulated by SEB and confirmed the previous report (15) whereas the same treatment did not alter phosphorylation of CD3-ζ in anergic $V\beta8^+$ T cells. Interestingly, in contrast to normal T cells, CD3-ζ was phosphorylated at a high level in anergic $V\beta8^+$ T cells prior to stimulation. Since CD3-ζ shifts its migration on SDS-PAGE from 16 Kd to 21 Kd upon phosphorylation (Baniyash, M., Garcia-Morales, P. Luong, E., Samelson, L. E., & Klausner, R. D. J. Biol. Chem. 263, 18225-18230. (1988)), the western blot filter loaded with the same sample was probed by CD3-ζ-specific antibody to confirm the presence of CD3-ζ protein (Fig. 11B). The results showed CD3-ζ protein at 16 Kd and further indicated that the bands in ptyr-specific western blots were phosphorylated CD3-ζ. The amounts of CD3-ζ protein co-precipitated with Vβ8-TCR were similar in control and anergic T cells regardless of their pre-activation with SEB. This observation was clearly confirmed in an experiment where larger amount of immuno-precipitated samples were studied (Fig. 11C).

The observation of constitutively phosphorylated CD3-ζ prompted investigation of the tyr-phosphatase in anergic $V\beta8^+$ T cells because this enzyme, represented to date by CD45, is known to be essential for responses to TCR stimulation (Koretzky, G. A., Picus, J., Schultz, T. & Weiss A. Proc. Natl. Acad. Sci. USA 88, 2037-2041. (1991)). The anergic $V\beta8^+$ T cells expressed levels of membrane CD45 equivalent to control cells. Furthermore,

when the membrane fraction of purified anergic Vβ8⁺ cells was assayed for total tyr-phosphatase activity it was indistinguishable from that of control cells and was equally sensitive to the action of specific phosphatase inhibitor, Na-orthovanadate (18) (Fig. 12). Therefore, the data document aberrant tyr-phosphorylation of TCR-associated CD3-ζ in anergic Vβ8⁺ T cells in the presence of normal functional phosphatase activity.

fyn is a member of *src* family of tyr-kinases and represents the only characterized PTK known to associate with TCR (Samelson,E. S. et al. Proc. Natl. Acad. Sci. USA 87, 4358-4362. (1990)). Whereas the functional role of *fyn* is not fully understood, both *fyn* and CD3-ζ appear to be crucial components for signal transduction through the TCR (Cooke, M. P., Abraham, K. M., Forbush, K. A.& Perlmutter, R. M. Cell 65, 281-291. (1991) and Irving, B. A.,& Weiss, A. Cell 64, 891-901. (1991)). It is therefore possible that abnormalities in *fyn or* CD3-ζ expression or action are responsible for the failure to transduce signals through the TCR in anergic Vβ8⁺ T cells. However, previous data indicated that anergic Vβ8⁺ T cells possess amounts of TCR-associated CD3-ζ equivalent to control cells and therefore it was postulated that *fyn* could be 1) decreased in absolute amounts; 2) unable to associate with TCR; or 3) unable to be activated by the TCR in anergic T cells. These issues were initially approached by performing western blot analysis of purified anergic Vβ8⁺ T cell extracts using specific antibody against *fyn* (Fig. 13). When *fyn* was probed in whole cell extracts of purified Vβ8⁺ T cells, the intensity of the bands was similar between control and anergic cells (Fig. 13A). A study of the *fyn* which co-precipitates with Vβ8-TCR further demonstrated that the amount of TCR-associated *fyn* is comparable between control and anergic T cells (Fig. 13B). Thus, an inadequate amount or insufficient association of *fyn* with TCR are unlikely explanations for

the anergic state. To test the possibility that the activation of TCR-associated *fyn* is aberrant in anergic T cells, the function of TCR-associated *fyn* was investigated by measuring the kinase activity of TCR-associated *fyn* in samples immuno-precipitated with anti-Vβ8 antibody from mild detergent (digitonin) cell lysates (Mustelin, T et al. Proc. Natl. Acad. Sci. USA 86, 6302-6306. (1989)). Activation of control cells with SEB dramatically increased TCR-associated *fyn*-kinase activity in anti-Vβ8 immuno-precipitates (Fig. 13C). In contrast, TCR-associated *fyn*-kinase activity increased only slightly following SEB activation of purified anergic Vβ8$^+$ T cells.

Thus the data demonstrated that TCR-associated *fyn* remains inactive following the stimulation of anergic T cells. The above described investigations have shown that TCR stimulation does not activate the TCR-associated *fyn* in anergic Vβ8$^+$ T cells. Thus, unresponsiveness to TCR stimulation of anergic T cells *in vivo* may be due to a defect (or defects) in activation of TCR-associated PTK(s). These cells, however, produce IL-2 (Fig. 9) and proliferate in response to co-stimulation with PMA plus ionomycin. They seem to carry a functional IL-2 gene which can be regulated by downstream events initiated by increases in cytosolic calcium and activation of protein kinase C (PKC). A similar phenomenon was observed previously when cells were treated with a PTK-inhibitor, Herbimycin A, in which TCR stimulation failed to induce IL-2 production. Co-stimulation with PMA plus ionomycin induced IL-2 production in the presence of Herbimycin A (Irving, B. A.,& Weiss, A. Cell 64, 891-901. (1991)). Blackman reported that TCR Vβ8.1 transgenic mice generated in an Mls-1^a carrying host develop anergic CD4$^+$, Vβ8.1$^+$ cells in the periphery (Blackman, M. R., Gerhard-Burgert, H., Woodland, D. L., Palmer, E., Kappler, J. W. & Marrack, P. Nature 345, 540-542. (1990)). These cells are unable to mobilize intracellular calcium when stimulated with a

TCR-specific antibody. However, similar to SEB-induced anergic Vβ8⁺ T cells, they proliferated in response to PMA and ionomycin (Blackman, M. A. et al. Proc. Natl. Acad. Sci. USA 88, 6682-6686. (1991)). Because calcium mobilization is triggered by inositol 1,4,5-triphosphate created by hydrolysis of inositol phospholipids by phospholipase C-γ (PLC-γ), and since a TCR-associated PTK activates PLC-γ by phosphorylation of tyr-residues (24), anergic T cells in transgenic mice may have defect(s) in the signaling circuit, such as TCR-associated PTK - PLC-γ - intracellular calcium release and/or PKC activation.

Anergic T cells have constitutively phosphorylated CD3-ζ. Interestingly, $CD4^-$, 8^- T cells of MRL*lpr/lpr* autoimmune-prone mice also respond poorly to TCR activation and also possess similarly constitutively phosphorylated CD3-ζ (Katagiri, K., Katagiri, T., Eisenberg, R. A., Ting, J. & Cohen, P. L. J. Immunol. 138, 149-156. (1987) and Samelson, L. E. et al. Nature 324, 674-676. (1986)). Similarly, $CD45^-$ cell lines fail to signal through the TCR (Koretzky, G. A., Picus, J., Schultz, T. & Weiss A. Proc. Natl. Acad. Sci. USA 88, 2037-2041. (1991)). However, CD45 levels and tyr-phosphatase activities were normal in SEB-induced anergic T cells. Taken together the observations suggest that persistent phosphorylation of TCR components may result in anergy in T cells. Of interest concerning this hypothesis is the observation that phosphorylation of terminal tyr-residue of *src* family tyr-kinases negatively regulates their enzyme activities (Davidson, D., Chow, L. M. L., Fournel, M. & Veillett, A. J. Exp. Med. 175, 1483-1492. (1992)). Therefore, constitutive tyr-phosphorylation of this residue of *fyn* may impair *fyn* dependent TCR signal transduction and result in T cell anergy.

Although *fyn* and CD3-ζ are essential for signalling, and are known to associate with the TCR, it is unclear which

component of the TCR is responsible for *fyn* association and whether this interaction requires additional molecules. Furthermore, it has been suggested that additional tyr-kinases are involved in the TCR signal transduction system (Chan, A. C., Irving, B. A., Fraser, J. D., & Weiss, A. Proc. Natl. Acad. Sci. USA 88, 9166-9170. (1991)).

Further details of the methods used in the above described investigations and the Figures illustrating the results of the investigations are set out below.

Figure 9

Figure 9 shows that anti-Vβ8 antibody treatment fails to generate inositol phosphates in SEB-anergized spleen T cell, but co-stimulation by PMA plus ionomycin induces IL-2 production in anergic Vβ8$^+$ T cells.

A. Total [^{3}H]inositol phosphates (cpm) represent the radioactivity derived from 5 x 10^6 cells. Control, PBS-primed spleen T cells; SEB-primed, 100 μg SEB-primed and anergic spleen T cells. This experiment was performed three times with similar results. B. CTLL-2 growth promotion by supernatants of SEB-stimulated Vβ8$^+$ T cells. C. CTLL-2 growth promotion by supernatants of PMA plus ionomycin stimulated Vβ8$^+$ T cells. In these assays supernatants of non-stimulated cells had some activity to promote CTLL-2 growth as twice diluted samples of control and SEB-primed Vβ8$^+$ T cells had 1200 cpm and 500 cpm respectively while CTLL-2 in medium alone had 200 cpm. Therefore, T cell activation owing to the antibody mediated positive purification did not compare with activation by SEB or PMA plus ionomycin that had 3-4 x10^4 cpm. D. Titration of IL-2 in supernatants of SEB or PMA plus ionomycin stimulated Vβ8$^+$ T cells. Open circles represent the data of control Vβ8$^+$ T cells and black circles represent the data of anergic Vβ8$^+$ T cells. Control, PBS-primed; Anergic, 100 μg SEB-primed; IL-2

Units, IL-2 units per ml. This experiment was performed three times with similar results.

METHODS.

A: Balb/c ByJ mice (4-6 weeks old) (Jackson Laboratory, Bar Harbor, ME) were given single intraperitoneal injections of 100 μg SEB (Sigma, St. Louis, MO) in 0.2 ml PBS to induce SEB-specific tolerance. In control mice, 0.2 ml of PBS was injected. Seven days later, spleen single cell suspensions were isolated and treated with Tris buffered 0.16 M Ammonium chloride to lyse red blood cells (RBC). T cells were enriched by depletion of surface immunoglobulin (Ig)-positive cells on goat anti-mouse Igs (100 μg/ml)-coated petri dishes ($5x10^7$ cells/10 cm dish). Nonadherent cells were further incubated with anti-rat Igs coated magnetic beads (cross-reactive with mouse Igs) (Dynabeads M450, DYNAL A.S., Oslo, Norway) and then were sorted by magnetically activated cell sorter (MACS, Miltenyi Biotec GmbH., Meitzfeld, West Germany). This process resulted in a negatively selected CD3-positive T cell population (~95% of $CD3^+$ cells, ~5% of $CD3^-$, Ig^- cells and ~1% of Ig^+ cells) and this population is referred to herein as spleen T cells. Accumulation of inositol phosphates in spleen T cells was measured by the reported method (Mizuguchi J. et al (1986) J. Immunol. 137, 2162-2167). SEB-primed or control spleen T cells ($2x10^7$/ml) were labelled with 40 μ Ci/ml myo-[2-^{3}H]-inositol (NEN, Boston, MA) for 5 hrs at 37°C in PBS containing 10 % PBS dialysed FCS. Cells were intensively washed and further incubated for 10 min in RPMI1640 medium supplemented with 10 mM LiCl ($5 x10^6$/ml). [^{3}H]-inositol labeled T cells were incubated at 37°C with biotinylated anti-Vβ8 antibody (5 μg/ml) for 15 min. Avidin (Sigma) was added to the reaction mixture (25 μg/ml) for the last 10 min to cross-link cell bound antibodies. The reaction was stopped by the addition of CH_3Cl/CH_3OH (1:2). The lysate was extracted with a 1:1 mixture of CH_3Cl/H_2O. The aqueous phase was applied to a 0.5 ml Dowex-1x8 (Bio-Rad,

Richmond, CA) and unbound materials were removed by washing with 5 mM myo-inositol and the total inositol phosphates were eluted with 1.5 ml of 0.1 M formic acid / 1.0 M-Na-formate and radioactivity was measured by β-counter. B, C, D: RBC-depleted spleen cells were incubated for 30 min on ice with biotinylated anti-Vβ8 antibody (F23.1) at 10 μg of antibody per 5×10^7 cells in 1ml. Cells were washed and mixed with Streptavidin-magnetic beads (Dynabeads M280, DYNAL) at 10 μl/10^5 cells for 30 min at 4°C. The magnetically coated cells were isolated by a magnetic field generated by MACS. Purification of positively coated cells was repeated three times. This process resulted in greater than 90% purity for the Vβ8$^+$ phenotype. To obtain bead-free purified cells, they were 37°C cultured in RPMI1640 containing 10 % fetal bovine serum for 16 hrs according to the manufacturer's instruction manual (Dynal). After the culture more than 80 % of the cells were free from beads by microscopic study. Bead detached cells were negatively collected by MACS and were cultured in 96 well tissue culture plates (4×10^4 / well) with the 10 μg/ml SEB (Sigma) in the presence of 4×10^3 murine B lymphoma, A20-2J (class II MHC$^+$) (Fig. 9B) or phorbol ester, PMA (Sigma) (10 ng/ml) and the calcium ionophore (A23187, Calbiochem, San Diego, CA) (0.5 μM) (Fig. 9C) for 48 hours. Supernatants were collected and dialysed against RPMI1640 medium for 48 hours to remove PMA and ionomycin. IL-2 activity of supernatants was measured by growth promoting activity on the IL-2 dependent cell line, CTLL-2, as described (Kawabe Y. and Ochi, A J. Exp. Med. 172, 1065-1070 (1990). To quantitate the amount of IL-2, supernatants were diluted serially as shown in the bottom scale of Fig. 9 B, C. Proliferation of CTLL-2 was indicated by the average values of incorporated [^{3}H]-thymidine (Amersham, Arlington Heights, IL) on quadruplicate samples. Standard deviation was generally less than 10 %. IL-2 units in supernatants were

calculated based on the data in Fig. 9B and 9C and shown in Fig. 9D. The units represent supernatant dilution that yielded 50 % of maximal CTLL-2 proliferation in control culture containing recombinant mouse IL-2 (Genzyme, Cambridge, MA). The unit of recombinant mouse IL-2 was provided by the manufacturer and was referred to in the experiment. Because the supernatants of SEB-stimulated anergic Vβ8$^+$ T cells showed CTLL-2 growth promotion activity below 50 % of maximal value, the unit was calculated by the simple proportion based on cpm value compared with 50 % of maximal value.

Figure 10

Figure 10 shows the failure to increase intra-cellular protein tyrosine phosphorylation in SEB-anergized spleen T cell with SEB or anti-Vβ8 antibody treatments.

A. Spleen T cells were stimulated with SEB. B. Spleen T cells were stimulated with anti-Vβ8 antibody. In the diagram of densitometry analysis of the autoradiograph, thick lines indicate stimulated cells and thin lines are for non-stimulated cells. Where only thick lines were shown, the data of non-stimulated cells are similar to that of stimulated cells. This experiment was performed four times with similar results.

METHODS.

Spleen T cells were prepared as mentioned for Figure 9 above. This purification method is based on the negative selection of T cells (positive selection and elimination of surface Ig positive cells) and therefore prepared spleen T cells are not pre-stimulated with membrane bound antibodies. Purified T cells were incubated for 2 min at 37°C with formaldehyde fixed A20-2J in either the presence or absence of 10 μg/ml SEB as mentioned in Fig. 9 (A). Alternatively, spleen T cells were incubated with the excess amount of biotinylated anti-Vβ8 antibody (5 μg/10^6 cells) for 30 min on ice in serum-free RPMI 1640 medium. Cells were washed and resuspended in 37°C medium containing

10 μg/ml avidin (Sigma) for 2 min (B). Incubations were terminated by adding lysis buffer [50 mM Hepes Ph 7.5 / 150 mM NaCl / 2mM EDTA / 10 μg ml^{-1} aprotinin (Sigma) / 10 μg ml^{-1} leupeptin (Sigma) / 1 mM PMSF (Sigma) / 500 μM sodium orthovanadate (Sigma)]. After 20 min of incubation on ice, nuclei were pelleted and supernatants were subjected to SDS-PAGE on 8 to 16 % gradient polyacrylamide gels (Novex, San Diego, CA). The fractionated proteins were transferred to Immobilon (Millipore, Bedford, MA) and the membrane was incubated 16 hours in 5 % BSA / 10 mM Tris Ph 8.2 / 140 mM NaCl / 0.01 % NaN_3 to block nonspecific protein binding. The blots were incubated with affinity purified ptyr-specific rabbit antibodies (30) for 2 hours. The specificity of the antibody used in this study has been demonstrated previously (Koch, CA et al Mol Cell. Biol. 9, 4231-4140n (1989)). The membranes were then incubated with 1 μCi of ^{125}I-protein A (Amersham) in 5 ml blocking solution for 30 min. The filters were washed and autoradiographed. The molecular weight scale was determined by the bands of standard proteins (BRL, Gaithersburg, MD). Since the proportion of $V\beta8^+$ T cells in anergic spleen T cells is about 80% of the control's, 20% more cells were used for protein extraction. Densitometry analysis of the autoradiograph was done by computerized densitometer (Molecular dynamics, Dunnydale, CA). Since the intensity of the bands had considerable variation, densitometry analysis was done on three separate molecular size sections as shown. Therefore, each section in the diagram had a different scale of arbitrary units that allowed us to compare the band intensity in detail.

<u>Figure 11</u>

Figure 11 shows the analysis of Vβ8-TCR-associated, CD3-ζ in anergic spleen T cells.

A. ptyr-specific western blots of Vβ8-TCR complex. In the diagram of densitometry analysis of the autoradiograph, thick lines indicate for stimulated cells and thin lines are for non-stimulated cells. Where only

thick lines are shown, the data of non-stimulated cells were similar to that of stimulated cells. B. and C. CD3-ζ-specific western blots of Vβ8-TCR complex. Arrows designate the tyr-phosphorylated CD3-ζ in A., and tyr-non-phosphorylated CD3-ζ in B. and C.. H and L in B. designate the heavy and light chains of Ig, respectively used for immunoprecipitation. CD-ζ specific antibody did not detect tyr-phosphorylated CD3-ζ by immuno-blotting even when sensitivity of immuno-blotting was increased by the enhanced chemi-luminescence detection system as shown in B. and similar observation was reported by others (Chan AC et al Proc, Natl. Acad. Sci. USA 88, 9166-9170 (1991). These experiments were performed four times with similar results.

METHODS.

Spleen T cells were prepared as mentioned above for Fig. 9 from normal and 100 μg SEB-primed mice and were stimulated with SEB for 2 min as described in Fig. 10 above. TCR was immuno-precipitated, according to previously described methods (Nakayama, T. et al Nature 341, 651-654 (1989)). Briefly, cells were lysed in 0.5 % Triton-X lysis buffer containing 5 mM EDTA and a $4x10^6$ cell equivalent extract (40 μl) was immuno-precipitated with Vβ8-specific antibody fixed on protein A conjugated beads (Parmacia, Uppsala, Sweden) at 4°C for 1 hour. Beads were washed and immuno-precipitated proteins were solubilized in loading buffer for SDS-PAGE. Immuno-precipitates were then fractionated on a 12 % SDS gel (Novex) and transferred to Immobilon and assayed by immuno-blotting for ptyr in Fig. 11A as described in Fig. 10. The bands shown at about 21 Kd were analyzed by densitometer as mentioned in Fig. 10. To confirm CD3-ζ protein associated with the TCR, the western blot filter loaded with the same immuno-precipitated proteins was probed by immuno-blotting with anti-CD3-ζ antibodies (Nakayama, T. et al Nature 341, 651-654 (1989)) followed by donkey anti-rabbit secondary antibody conjugated with horseradish peroxidase (Amersham)

and protein blots were developed using the enhanced chemiluminescence detection system (ECL) (Amersham) (Fig. 11B). In Fig. 11C, $2x10^7$ cell equivalent Triton-X lysis buffer extract (200 µl) was studied similarly to Fig. 11B, but CD3-ζ protein was probed with CD3-ζ-specific antibody and ^{125}I-protein A.

Figure 12

Figure 12 shows the phosphatase activity of anergic $V\beta8^+$ T cells. Total membrane fraction was examined for the tyr-specific phosphatase activity. Circles indicate samples prepared from purified normal $V\beta8^+$ T cells while squares are that of purified anergic $V\beta8^+$ T cells. Assays were done in the presence (filled symbols) and absence (open symbols) of 1mM Na_3VO_4. All experiments were performed independently and in triplicate and were performed three times with similar results.

METHODS.

$V\beta8^+$ T cells were purified as mentioned above for Fig. 9 and membrane fractions were prepared according to the method by Mustelin (Proc. Natl. Acad. Sci. USA 86, 6302-6306 (1989)). Briefly, MACS-purified $V\beta8^+$ T cells from PBS or SEB (100 µg) treated spleen cells were immediately sonicated in a hypotonic lysis buffer [25 mM Tris-Hcl Ph 7.5 / 25 mM sucrose / 0.1 mM EDTA / 5 mM dithiotreitol / 1 mM phenylmethylsulfonyl fluoride (PMSF) / leupeptin (10 µg/ml) / aprotinin (10 µg/ml)], in the presence and absence of 1 mM Na_3VO_4. After nuclei were removed by low-speed centrifugation, the membrane was sedimented at 100,000 g for 60 min at 4°C. The resulting pellet was suspended in lysis buffer by sonication. Protein concentration was determined with the protein assay kit (Bio-Rad, Richmond, CA). The phosphatase activity was measured according to previously described methods (Zanke B et al Eur. J. Immunol. 22, 235-239 (1992)). Briefly, 5-20 µg of membrane protein was incubated in a reaction mixture of 5 mM P-nitrophenyl phosphate (Sigma), 80 mM 2-[N-morpholino]ethane sulfonic acid (Ph 5.5), 10 mM EDTA,

and 10 mM DTT, at 37°C for 15 minutes. The reactions were stopped by the addition of 0.2 N NaOH. Absorbance was measured at 410 nm and expressed for each value as a percentage of maximally observed.

Figure 13

Figure 13 shows the analysis of CD3-ζ and *fyn* proteins, and *in vitro* kinase function in Vβ8$^+$ T cells.A. Total *fyn* of Vβ8$^+$ T cells. B. TCR-associated *fyn*. Since Balb/c mice spleen T cells are negative for Vβ3$^+$ T cells due to thymic selection, immunoprecipitation with anti-Vβ3 antibody provides a negative control for anti-Vβ8 antibody. The final lane represents a spleen T cell lysate to indicate the total amount *fyn* present in an equivalent number of cells. C. *In vitro* kinase assay of Vβ8 TCR-associated PTK. In the densitometry analysis of data labeled as control and anergy, thick lines indicate for stimulated cells and thin lines are for non-stimulated cells. Where only thick lines were shown, the data of non-stimulated cells were similar to that of stimulated cells. *fyn, lck, C : After the kinase reaction was performed with immuno-precipitated TCR, the immune complex from SEB-activated control cell was incubated in 1 % SDS at 95°C for 10 min and diluted 10 fold in buffer with 1 % Nonidet P-40. The samples were re-immuno-precipitated with either anti-*fyn*, anti-*lck* (Veillette, A. et al Cell 55, 301-308 (1988)), or control normal rabbit antibodies as indicated. Arrows designate the position of *fyn* protein. These experiments were performed four times with similar results.

METHODS.

A. Vβ8$^+$ T cells were purified as mentioned above for Fig. 9 and were suspended in lysis buffer containing 1 % NP40. Cell extracts were separated on a 8 % polyacrylamide gel (Novex) and transferred to Imobilon. Protein blots were hybridized with anti-*fyn* (1:100 diluted antiserum) (Veillette, A. et al Cell 55, 301-308 (1988)) followed by ^{125}I-protein A. B. Spleen T cells were prepared as

mentioned above for Fig. 9. Cell extracts in 1 % digitonin were immune-precipitated with anti-Vβ8 or anti-Vβ3 (KJ-25) (Pullen AM et al Nature 335, 796-801 (1988)) antibodies. Purified TCR complexes were resolved on a 8 % SDS gel and transferred to Imobilon. The filter was incubated in blocking solution (5 % BSA) for 16 hours, and further incubated with anti-*fyn* antibody for 2 hours at room temperature. The protein blots were developed using the ECL system (Amersham). C. Spleen T cells were prepared as mentioned above for in Fig. 9. Cells were stimulated with SEB as mentioned in Fig. 9 and solubilized at 10^7 /ml cells in 50 mM Hepes / 150 mM NaCl / 2 mM EDTA / 1 % digitonin (w/v), 10 μg ml^{-1} aprotinin / 10 μg ml^{-1} leupeptin / 1 mM PMSF / 0.5 mM Sodium orthovanadate. The lysates were centrifuged at 12 $x10^3$ g for 10 min and supernatants were pre-cleared for 30 min with 50 μl of 10 % Staphylococcus Cowan' Strain I (Calbiochem). The supernatants were then incubated with anti-Vβ8 antibody for 1 hour at 4°C. The immunocomplex was then collected by incubation with Protein A-Sepharose 4B (Pharmacia) 4°C for 30 min. After immunoprecipitation, the immunocomplex was washed three times in lysis buffer without EDTA and 3 times with kinase buffer (50 mM Hepes Ph 7.5 / 100 mM NaCl / 5 mM $MnCl_2$ / 5 mM $MgCl_2$ / 1 μM ATP) and suspended in a 50 μl of kinase buffer containing 10 μ Ci of γ-^{32}P-ATP (Amersham) (3 x 10^3 Ci / mM). After 15 min at 30°C, the reaction was terminated by the addition of an equal volume of 2 x SDS sample buffer (20 % glycerol, 10 % 2-mercaptoethanol, 4.6 % SDS, 125 mM Tris Ph 6.8, 0.004 % Bromophenol blue). The samples were boiled and analyzed by electrophoresis through a 8 % SDS-polyacrylamide gel. Densitometry analysis of autoradiograph was done similarly to Fig. 10.

Example 5

This example describes an investigation of the efficacy of Staphylococcus aureus enterotoxin B (SEB) coupled to tumor

specific anti-idiotypic antibody in redirecting T cell effector activity to the growth inhibition of B lymphoma 38C13 cells. Incubation of 38C13 lymphoma cells with syngeneic C3H/He splenic cells and SEB-anti-idiotype (Id) conjugate was associated with between 80 and 100% growth inhibition of the tumor cells. Intravenous administration of SEB-anti-Id to mice previously inoculated with 38C13 lymphoma cells led to 40%< survival at 100 days compared to a mean survival of 21 days in control animals. When this reagent was compared with other targeting constructs - the anti-CD3-anti-Id and anti-T-cell receptor (TCR) Vβ8-anti-Id - they were more or less effective to prevent tumor growth. However, anti-CD3-anti-Id impaired almost the entire T cell response whereas the effects of SEB- or anti-Vβ8-anti-Id had effects limited to Vβ8 T cells. These results - together with the fact that *in vivo* administration of SEB causes a small change in $V\beta8^+$ T cell numbers contrasting to anti-Vβ8 antibody, which depletes them - suggest that SEB-anti-tumor antibody conjugates represent a potentially powerful approach for better tumor immunotherapy.

The following materials and methods were used in the investigation summarized above.

Mice.

C3H/ He mice ($H-2^k$) 6-8 weeks old were obtained from The Jackson Laboratories, Bar Harbor, ME.

Conjugates preparation.

The anti-Id (S5A/2b) (Maloney, D. G., M. S. Kaminski, D. Burowski, J. Haimovich, and R. Levy. 1985. *Hybridoma* 4:191), anti-Vβ8 (F23.1) (Staerz, U. D., H-G. Rammensee, D. J. Benedetto, and J. M. Bevan. 1985. *J. Immunol.* 134:3994), and anti-CD3ε (2C11) (Leo, O., M. Foo, D. H. Sachs, L. E. Samelson, and J. A. Bluestone. 1987. *Proc. Natl. Acad. Sci. USA* 84:1374) antibodies were purified

from the culture supernatants of B cell hybridoma maintained in Dulbecco's medium supplemented with 10% of foetal bovine serum using protein G Sepharose (Pharmacia LKB, Uppsala Sweden) column. SEB-anti-Id, anti-Vβ8-anti-Id, and anti-CD3-anti-Id complexes were prepared by using SPDP according to the manufacture's handbook (Pharmacia). Aliquots of 2 to 5 mg of SEB and antibodies in 5 ml 0.1 M Na Phosphate, 0.1 M NaCl (Ph 7.2) buffer were incubated for 2 hrs at 37°C with an 8 molar excess of SPDP. The SEB, anti-Vβ8, or anti-CD3ϵ were dialyzed against 0.1 M Na Phosphate, 0.1 M NaCl (Ph 7.2) buffer at 4°C, whereas the anti-Id was dialyzed against 0.1 M Na-acetate, 0.1 M NaCl (Ph 4.5) at 4°C. Dithiothreitol (Sigma) was added to the SEB, anti-Vβ8 or anti-CD3ϵ antibody solution to a final concentration of 0.05 M and allowed to stand 30 min at room temperature. This solution was eluted on a Sephadex G50 gel filtration column (Pharmacia). The SEB, anti-Vβ8, or anti-CD3ϵ containing fractions were mixed with the anti-Id solution and incubated at room temperature for 4 hrs. The reaction was stopped by the addition of 1 mg iodoacetamide (BDH Chemicals Ltd., Poole, England) and the crosslinked material was eluted on an Ultrogel AcA34 column (Pharmacia LKB). Cross-linked material was separated from monomers, pooled and concentrated by using CF25 ultracentrifugation membrane cones (Amicon, Denvers, MA). The concentration of the conjugate was determined by Boi-Rad Protein Assay (Bio-Rad, Richmond, CA). The purity was determined by SDS-PAGE.

<u>*In vitro* assay for tumor growth.</u>

Growth inhibition by SEB-stimulated spleen cells.

Spleen single cell suspension of unprimed C3H/He mice was treated with Tris buffered 0.16 M Ammonium chloride to lyse the red blood cells (RBC). Varied doses of spleen cell were cultured with 10 μg/ml SEB in 96 multi-well U bottom plate. After 48 hrs of incubation at 37°C, the culture plate was irradiated (3000R) and 5 x 10^2 of 38C13

B lymphoma cells (H-2^k, class II MHC$^-$) (25) were added to each well. Fresh spleen cells were similarly RBC depleted, irradiated and co-cultured with 5 x 10^2 of 38C13 in the same doses of SEB-stimulated spleen cells in control cultures.

Growth inhibition by the pretreatment with anti-Id-SEB. 38C13 B lymphoma cells (2 x 10^6) were incubated on ice with 50 µg of anti-Id-SEB (in 50 µg PBS) in 1 ml of RPMI1640 medium containing 10 % FCS. The 50 µg of anti-Id alone, 50 µg SEB alone and the mixture of 25 µg anti-Id and SEB were used instead of the anti-Id-SEB conjugate for controls. Cells were washed and 5 x 10^2 cells were mixed with 10^5 or 10^6 RBC depleted, irradiated (3000R) fresh spleen cells in each assay well.

After 16 hrs of culture, cells were pulsed with 1 µCi/well of [^{3}H]-Thymidine (Amersham, Buckinghamshire, England). Cells were collected 3 hrs later by glass fibre filter mats. Radioactivity was determined in a liquid scintillation β counter. The percentage of cell growth inhibition was calculated from average CPM values of quadruplicate samples as 100 x [1 - (co-culture of 38C13 and spleen cells - spleen cells alone)/(38C13 alone)].

Fluorocytometry.

Spleen single cell suspensions were treated with Tris buffered 0.16 M Ammonium chloride to lyse the RBC. These cells were double treated with FITC-anti-Thy1.2 (Sigma Chemical Co.) and biotinylated anti-Vβ8 (F23.1) or anti-Vβ6 (RR4-7) (26). Cells were washed and stained with Avidin-PE (Becton Dickinson & Co). Cells were washed followed by two-color (red and green) fluorescence analysis by Coulter Epics-C fluorocytometry. All incubations during cell staining were done on ice.

Statistical analysis of mice survival time.

Differences of the occurrence of tumor-free mice in anti-Id-SEB treated group and that of other control groups in the series of the experiments were analyzed by the median χ^2 test for significance, based on the null hypothesis that the percent of survivors for each treatment group was the same.

Details of the methods and results of the investigations summarized above are set out below.

A. In vitro effects of SEB-anti-Id to B lymphoma, 38C13.

To test the efficacy of SEB-anti-Id conjugates in tumor cell growth inhibition, a highly tumorigenic B cell lymphoma line, 38C13, was utilized which represents a carcinogen-induced tumor that is membrane IgM positive and class II MHC negative. The anti-idiotypic antibody used in the targeting construct was a mouse IgG2b monoclonal antibody, S5A/2b, specific for the 38C13 idiotype. In initial studies, the effects of SEB-stimulated T cells on tumor cell growth *in vitro* was assessed using co-cultures of irradiated SEB-activated C3H/He spleen cells with 38C13 cells. While incubation with fresh (untreated) spleen cells appeared to accelerate proliferation of the 38C13 cells, tumor growth was significantly inhibited by incubation with SEB-activated cells (Fig. 14a). To ascertain whether the SEB-anti-Id conjugate also inhibited lymphoma cell proliferation, experiments were repeated by culturing spleen cells with 38C13 cells after pre-incubation with conjugate. As shown in Figure 14b, incubation of irradiated fresh spleen cells and conjugate preincubated 38C13 cells led to 80 - 100% growth inhibition of the tumor cells. Pre-incubation with SEB or anti-Id was associated with insignificant growth inhibition. These results suggest that SEB induces activation of T cells that can inhibit tumor growth and thus that SEB-anti-idiotypic antibody conjugates are of

potential value in the treatment of lymphomas.

B. *In vivo* effects of SEB-anti-Id to B lymphoma, 38C13.

The above mentioned results prompted an investigation of the efficacy of the SEB-based targeting construct in the elimination of 38C13 lymphoma cells *in vivo*. For these studies, C3H/He mice were injected intraperitoneally with 10^3 38C13 cells and the SEB-anti-Id conjugate was administrated intravenously 6 hours later. Inoculation of either SEB or anti-Id alone into 38C13-inoculated mice did not markedly alter the mean survival time (21 days) from that observed in control mice inoculated with 38C13 cells and PBS (Table 7). By contrast, 40% of mice treated with SEB-anti-Id conjugates survived for at least 100 days after tumor inoculation and could thus be regarded as tumor free (χ^2 =12.5, $P<0.005$). Treatment with conjugates of an irrelevant antigen, ovalbumin, and anti-Id antibody unchanged survival time compared to that produced by controls (data not shown). Thus, the therapeutic benefit of SEB-anti-Id conjugate in the *in vivo* treatment of 38C13 lymphoma appears to be specifically related to the T cell stimulating properties of SEB.

C. Comparison of SEB-anti-Id with anti-CD3- or anti-Vβ8-anti-Id conjugates *in vivo*.

In order to evaluate SEB-anti-Id conjugate in comparison with other T cell targeting structures, antibody heterodimers, anti-CD3-anti-Id and anti-TCR Vβ8 (anti-Vβ8)-anti-Idwere constructed. The former virtually stimulates all peripheral T cells while the latter stimulates only Vβ8-TCR expressing cells (~30 % of T cells) which cover most of the SEB reactive cells (White, J., A. Herman, A. M. Pullen, R. Kubo, J. W. Kappler, and P. Marrack. 1989 *Cell* 56:27; Choi, Y., B. Kotzin, L. Herron, J. Callahan, P. Marrack, and J. Kappler. 1989. *Proc. Natl. Acad. Sci. USA* 86:8941,Kawabe, Y., and A. Ochi. 1990 *J. Exp. Med.* 172:1065; and Kawabe, Y., and A.

Ochi. 1991 *Nature (London)* 349:245.). When these conjugates were tested for the efficacy to prevent the 38C13 growth *in vivo* anti-CD3-anti-Id was found to be ineffective to develop survivors whereas SEB-anti-Id and anti-Vβ8-anti-Id produced tumor-free animals (Table 8). This observation may suggest that anti-CD3-anti-Id is a weaker reagent than others for *in vivo* application. In order to examine the efficacy of anti-CD3-anti-Id in more detail a 38C13 sub-clone which grows slower than original cell (doubling time is about 1.5 times longer than that of wild type cell) were targeted in a similar protocol (Table 9). In this experiment both anti-CD3-anti-Id and SEB-anti-Id were effective to develop tumor free animals while the anti-CD3 or anti-Id alone treated group developed 10-20 % of survivors. Thus, data indicated that all three constructs had more or less the effect of preventing the *in vivo* growth of 38C13 lymphoma but suggested that anti-CD3-anti-Id had less efficacy to prevent tumor growth *in vivo* than others.

D. Effects to T cell response in targeting reagents treated mice.

It was decided to examine whether 10-day pre-injection of TCR-specific antibodies and superantigens influenced the T cell mitogenic response in mice since TCR-specific antibodies and superantigens have been reported to modulate T cell responses *in vivo* (Thistlethwaite Jr., J. R., B. A. Cosimi, L. F. Delmonico, H. R. Rubin, N. Talkoff-Rubin, W. P. Neilson, L. Fang, and P. Russel. 1984 *Transplantation* 38:695; Jaffers, J. G., C. T. Fuller, B. A. Cosimi, S. P. Russell, J. H. Winn, and B. R. Colvin. 1986 *Transplantation* 41:572; Ellenhorn, D. I. J., R. Hirsch, H. Schreiber, and J. A. Bluestone. 1988 *Science* 242:569; Hirsch, R., M. Eckhaus, D. H. Sachs, and J. A. Bluestone. 1989 *J. Immunol.* 140:3766; White, J., A. Herman, A. M. Pullen, R. Kubo, J. W. Kappler, and P. Marrack. 1989 *Cell* 56:27; Kawabe, Y., and A. Ochi. 1990.

J. Exp. Med. 172:1065, Kawabe, Y., and A. Ochi. 1991. *Nature (London)* 349:245; Rellahan, B. L., L. A. Jones, A. M. Kruisbeek, A. M. Fry, and L. A. Matis. 1990 *J. Exp. Med.* 172:1091; MacDonald, H. R., S. Baschieri, and R. K. Lees. 1991 *Eur. J. Immunol.* 21:1963; Kim, C., K. A. Siminovitch, and A. Ochi. 1991 *J. Exp. Med.* 174:1431.27-30), As shown in Figure 15, both SEB and anti-Vβ8 conjugates reduced SEB-specific proliferative response. In contrast with these groups anti-CD3-conjugate treated mice lost response to all the stimulating reagents *in vitro*. Furthermore, in spite of the similar effect by SEB and anti-Vβ8 modified reagents, the Vβ8$^+$ T cells were depleted in anti-Vβ8 treated mice while SEB decreased the proportion of Vβ8$^+$ T cells slightly (Table 10 and Kawabe, Y., and A. Ochi. 1991 *Nature (London)* 349:245; MacDonald, H. R., S. Baschieri, and R. K. Lees. 1991 *Eur. J. Immunol.* 21:1963; Zaller, D. M., G. Osman, O. Kanagawa, and L. Hood. 1990 *J. Exp. Med.* 171:1943). Thus, TCR-specific antibody therapy resulted in the depletion of reactive cells *in vivo* while the superantigen treatment had a slight effect on the total cell number in the periphery.

Overall, these results show that superantigens such as SEB represent a potent targeting reagent when conjugated to tumor-specific antibody. Although activation of T cells by SEB in the conjugate requires class II MHC antigen, the tumor targets are not necessary to express it and thus SEB-mediated targeting may be applicable to treatment of a wide variety of tumors (Fig. 16). Recently, Newell et al. showed that SEB-alone injection prevents outgrowth of subcutaneously inoculated skin tumor cells in mice (Newell, K. A., J. D. I. Ellenhorn, D. S. Bruce, and J. A. Bluestone. 1991 *Proc. Natl. Acad. Sci. USA.* 88:1074). However, the present inventor did not observe significant effect to induce more survivors by SEB-alone injection. The difference may be because of the different

malignancies of tumors, as skin tumor did not grow in about 25 % of non-treated recipients but 38C13 killed all mice within 25 days after injection of 10^3 cells in the present inventors experiments. Be that as it may, the reported results suggest that bacterial superantigens are beneficial for immunotherapy of cancer. Moreover, since the different Staphylococcus enterotoxins (SEs) are specific for different Vβ families (A. Herman, J. W. Kappler, P. Marrack, and A. M. Pullen. 1991. SUPERANTIGENS: Mechanism of T cell stimulation and role in immune responses. *Annu. Rev. Immunol.* 9:745) (eg. Vβ7,8.1,2,3 respond to SEB but Vβ1,3,10,11,12,17 respond to SEA) it should be possible to control the proportion of T cells invoked in the targeting system by using different combinations of SEs in the conjugate.

The results also provided an important insight that *in vivo* immunotherapy of cancer results in better consequences when a part of T cells were used rather than entire T cells.

The Figures illustrating the investigations in Example 5 will be described in more detail below.

Figure 14.

1a.Growth inhibition of 38C13 B lymphoma by SEB-stimulated spleen cells.

1b.The pre-treatment of 38C13 B lymphoma with anti-Id-SEB inhibit the growth of 38C13 in co-culture with spleen cells.

In all experiments, the cell growth of the irradiated spleen cells was less than 10 % of 38C13 cells. Standard deviation was normally less than 10%. The data are representative of three experiments.

Figure 15. The effect of injection of targeting reagents on peripheral T cell function.

C3H/He mice were injected intravenously with 50 µg of SEB-anti-Id, anti-CD3-anti-Id, or anti-Vβ8-anti-Id in 0.2 ml PBS. Ten days later spleen single cell suspensions (10^5/well) were stimulated *in vitro* in 96 well U bottom plate with T cell mitogenic reagents anti-CD3 (10 µg/ml); SEA (2 µg/ml); SEB (10 µg/ml). After 48 hrs of culture, cells were pulsed with 1 µCi/well of [^{3}H]-Thymidine for 12 hrs.

* ; Spleen cells were stimulated *in vitro* with purified anti-CD3 (10 µg/ml), SEA (2 µg/ml), SEB (10 µg/ml).

** ; Mice were injected with 50 µg of these reagents 10 days before assay.

Figure 16. Targeting of B lymphoma (Class II MHC$^-$, Id$^+$) with SEB-anti-Id.

SEB-conjugated anti-Id antibody binds to surface Ig of B lymphoma. Juxtaposed antigen presenting cells (APC)s such as B cells and macrophages bind to SEB to make SEB-Class II MHC complex. This complex stimulates T cells (represented by CD4$^+$ T cell in the schema) expressing Vβ7 or Vβ8.1,2,3 TCRs. Activated T cells deliver growth inhibition to B lymphoma cells.

Example 6

The possible physiological relevance of clonal anergy and death of T cells in vivo was investigated.

A. Unresponsiveness of CD4$^+$, Vβ8$^+$ T cells is reversible in vivo.

In order to investigate if anergic T cells are terminally differentiated, Staphylococcal enterotoxin B (SEB)-specific T cell anergy was induced in thymectomized mice and the response to SEB was monitored at different times. As in normal mice, thymectomized mice became SEB-specific tolerant and the proportion of CD4$^+$, Vβ8$^+$ T cells was reduced to 60 % of control in spleens when examined on day

10 post SEB priming. Both proliferation and interleukin 2 (IL-2) production by spleen cells were suppressed in SEB-primed mice to less than 30 % of PBS-primed control mice. The response to another superantigen Staphylococcal enterotoxin A (SEA) was comparable in both PBS-primed and 50 μg SEB-primed mice. When spleen cells were assayed in vitro for their SEB-specific proliferative response 2 months after SEB-priming, the response was higher than 10 days post SEB-priming but was still below that of control spleen cells. Thus mice were apparently recovering from tolerance 2 months after SEB challenge. Four months later SEB-primed spleen cells responded efficiently to SEB and the cell dose dependent response curve was equivalent to that of control spleen cells. IL-2 production of SEB-stimulated spleen cells also recovered to the level of controls. The recovery of the proliferative response to SEB was further demonstrated when $CD4^+$, $V\beta8^+$ T cells of SEB-primed and control mice were purified and their response determined. The proliferation and IL-2 production to different dosages of SEB were identical between recovered and normal cell samples but the percentage of $V\beta8^+$, $CD4^+$ or $CD8^+$ T cells on day 10 was maintained even after 4 months and there was no increase in the number of $CD4^+$, $V\beta8^+$ spleen T cells. The study indicates that unresponsiveness of $CD4^+$, $V\beta8^+$ T cells is reversible and after four months cells regain a proliferative response comparable to that of control mice.

B. A protein synthesis inhibitor, cycloheximide (CX) blocked anergy induction but not death of $V\beta8^+$ T cells when injected with SEB.

Whereas the molecular change which induces the anergy of T cells is not clear yet, a report showed that de novo protein synthesis was essential for T cell unresponsiveness in vitro (Quill & Schwartz 1987 J. Immunol. 138, 3704-3712). In order to understand the

molecular mechanism of SEB-specific $CD4^+$, $V\beta8^+$ T cell anergy in vivo the effect of CX on tolerance induction was tested. A sublethal dose of CX was injected i.p. with SEB into mice at different times. The tolerance to SEB stimulation was examined after 10 days. The results demonstrated that in vivo tolerance induction to SEB was dependent on de novo protein synthesis. The effective period was only within the first 12 hours and after that period the injection of CX did not prevent anergy. The duration of CX-induced inhibition of protein synthesis in vivo was determined by measuring the time required for resumption of protein synthesis in spleen after 1mg CX injection by pulsing mice with [^{35}S]-methionine. After 6 hours de novo protein synthesis equivalent to normal animals was detected. Probably owing to this short lasting effect by CX in vivo, expansion and deletion of $V\beta8^+$ T cells equivalent to normal mice were associated with all animals with CX despite their lack of specific tolerance. Therefore protein synthesis important for anergy induction seemed to take place within 12 hours after SEB injection. The results also suggested that activation induced cell death was able to take place in mice despite the prevention of anergy. This may indicate that anergy is not a conditional process for cell death. It is also probable that anergy and cell death are mechanistically differential. Current information does not clarify if the prevention of T cell anergy induction is because of protein synthesis inhibition in responding T cells or in other cellular components like antigen presenting cells (APC). However SEB does not require processing by APC to stimulate T cells (Janeway et al. 1989 Immunol. Rev. 107, 61-88) and the TCR β-chain spontaneously binds to high affinity complexed SEB and class II MHC. Therefore the inhibition of anergy by CX is most probably because of the inhibition of protein synthesis essential for anergy in $CD4^+$, $V\beta8^+$ T cells in vivo.

C. DNA degradation occurs exclusively in T cell blast of SEB-primed spleen.

The results obtained in the studies herein showed that the clonal deletion of reactive $V\beta^+$ T cell took place only after the initial expansion of the same $V\beta$ phenotype cells. The results suggested that programmed cell death (PCD) occurs in proliferating cells and its development depends on cellular activation signals. Similarly, results of studies on PCD in T cell hybridomas linked this phenomenon with cell activation (Mercep et al. 1988 J. Immunol. 140, 324-335, Ucker et al. 1989 J. Immunol. 143, 3461-3469). These cells expressed increased amount of IL-2R. To test that deletion occurred in activated cells in our system, cell death was compared between density gradient separated blastic and non-blastic spleen cells obtained from SEB-primed mice 3 days after SEB injection. DNA fragmentation was observed exclusively in the blastic cell fraction as is consistent with the contention that cells targeted for clonal deletion undergo activation and blastogenesis prior to their PCD in vivo.

D. The T helper (Th) clones become unresponsive without proliferation in vitro.

The functional unresponsiveness of T lymphocyte has been reported by Schwartz using in vitro cultured protein antigen-specific Th cell clones (Schwartz 1990 Science 248, 1349-1356). In those, functional unresponsiveness was observed on Th clones which were in vitro antigen stimulated with chemically fixed APC, antigen and purified class II MHC molecules inserted into planar membranes, or immobilized CD3 complex-specific antibodies (Quill & Schwartz 1987 J. Immunol. 138, 3704-3712, Jenkins & Schwartz 1987 J. Immunol. 144, 16-22, Jenkins et al. 1988, J. Immunol. 140, 3324-3330; Jenkins et al. 1990 J. Immunol.144, 16-22). These experiments suggested the induction of protein antigen-specific unresponsiveness of

Th cells was the the result of occupancy of TCR in the absence of co-stimulatory signals (Mueller et al. 1989 Ann. Rev. Immunol. 7, 445-480). The anergic Th cell clones expressed unchanged levels of TCR and IL-2R, and were responsive to exogenous IL-2. Antigen stimulation could not induce autocrine production of IL-2 because of defective transcription of the IL-2 gene in these tolerant cells. In these studies the cells enlarged but other activation changes such as IL-2R up-regulation and thymidine incorporation were not observed (Quill & Schwartz 1987 J. Immunol. 138, 3704-3712, DeSilva et al. 1991 J. Immunol. 147, 3261-3267). Thus the data indicated that Th clones become anergic without cell division.

E. Anergic cells die.

In contrast to the above mentioned results which support the idea that anergic T cells are long-lived there is some evidence that anergic T cells are dying in vivo. An observation that we believe supports the proportion of $CD4^+$, $V\beta8^+$ cells continues to decline after SEB-stimulation at a time when these cells are already tolerant. This occurs in the period when $CD4^+$, $V\beta8^+$ T cell proportion is gradually decreasing after a major reduction between day 2 and 3 post SEB injection. This type of cell death may be taking place on a small scale even before this period but may be difficult to detect in this period of intense activity. It is an intriguing issue if these cells are slowly dying because of exhaustion from the strong activation. In any case it seems to be the case that these cells lose responsiveness before they die.

EXAMPLE 8

Polystyrene microtiter wells were coated with double-strand DNA (100 μg/ml 4°C 16 hours) to prepare assay plates. Blood from each mouse was pooled according to treatment group and collected before the biweekly injection. Sera was diluted in 0.05% Tween 20 in PBS at a 1:500 dilution and allowed to incubate on the plates for

60 min. at room temperature. The plates were then washed three times with PBS-Tween, and 50 µl of a 1:1000 dilution of anti-IgG and anti-IgM goat anti-mouse urease conjugate was added to the plates. After incubation for 30 min., the plates were washed three times with PBS Tween and twice with 0.15 M NaCl. The plates were then incubated with the substrate solution, and colour changes were assayed by Microplate Reader as mentioned. The results are shown in Fugure 17. In Figure 17(1), MRL-lpr/lpr mice (female) were given bi-weekly intravenous injections of 50 µg SEB beginning at 8 weeks of age. The date represent the blood samples from 18 weeks of mice (average of 6 mice). In Figure 17(2), NZB/NZW F1 mice (female) were given bi-weekly intravenous injection of 20 µg SEB beginning at 10 weeks of age. The data represent the blood samples from 36 weeks of mice (average of 5 mice).

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the invention.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the invention.

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Table 1. *Proteinuria*

Age	Treatment			
	50 μg SEB	5 μg SEB	0.5 μg SEB	PBS
wk		*g/liter*		
12	<0.3	<0.3	1.0	1.0
13	<0.3	<0.3	1.0	1.0–3.0
14	<0.3	<0.3	1.0	1.0–3.0
15	<0.3	0.3–1.0	1.0*	1.0–3.0
16	<0.3	0.3	1.0	1.0–3.0
17	<0.3	1.0	1.0–3.0	1.0–3.0
18	0.3–1.0	0.3–1.0	3.0	3.0‡
19	0.3–1.0	0.3–1.0	3.0–20‡	3.0
20	0.3	1.0–3.0	3.0–20‡	3.0
21	0.3–1.0	3.0–20‡	3.0–20§	3.0–20§

Measured from 3 mo of age (by Labstix).
* Trace, nonhemolyzed blood.
‡ Hemolyzed blood.
§ Large blood.

Table 2. *Physical Symptoms (Lymphoid Hyperplasia, Necrosis of Ear, Hair Loss)*

	Treatment			
Age	50 μg SEB	5 μg SEB	0.5 μg SEB	PBS
wk				
12	0	0	0	0
13	0	0	0.3	0.3
14	0	0	0.3	0.3
15	0	0	0.35	0.4
16	0	0	0.45	0.61
17	0	0.1	0.85	0.67
18	0	0.1	1.1	0.72
19	0	0.31	0.85	0.61
20	0	0.31	0.85	1.0
21	0.1	0.44	0.95	1.06

No symptoms, 0; trace, 0.5. Symptoms scored 1–4, (4 most severe). Total score for each group is divided by total number of mice.

Table 3

SEB but not TSST-1 induces specific T cell anergy *in vivo*

In vivo Treatment	Proliferative response (CPM) *in vitro* stimulated with PBS	SEB (10 μg/ml)	TSST-1 (5 μg/ml)
Control	977±288	17023±1173	25199±626
TSST-1 5μg	2301±491	17820±637	27636±133
TSST-1 50μg	1739±265	19187±1756	25491±626
TSST-1 100μg	1776±50	16241±2354	25521±1604
TSST-1 200μg	1586±82	19233±1320	30774±1863
SEB 50μg	1295±67	5333±321	23002±332

Balb/c mice were injected i.p. with various doses of TSST-1 or 50 μg of SEB. Twelve days later spleen cells ($4x10^4$) were assayed for the proliferative response to TSST-1 or SEB by the methods described before (32).

Table 4

The selected characteristics of SEA, SEB, and TSST-1

	SEA	SEB	TSST-1
Molecular size	27,800	28,300	24,000
T cell stimulation efficacy (22)	SEA > SEB > TSST-1		
Affinity to class II MHC (22)	SEA > SEB > TSST-1		
Preference of class II (22) MHC isotype	$A \geq E$	$A \leq E$	$A >> E$
TCR-Vβ specificity (27)	1,3,10,11,12,17	7,8.1,8.2,8.3	15,16
Activation induced PCD of T cells	Yes (Unpublished data)	Yes	Yes
Anergy	Yes	Yes	No
Tolerance	Yes	Yes	No
Major symptoms *in vivo*	Food poisoning	Food poisoning	Toxic shock

Table 5

Phenotype of anergic and awakened CD4$^+$, Vβ8$^+$ T cells.

Phenotypes	% positive cells			
	10 days		4 months	
	Control*	SEB**	Control	SEB
Ly-24	92.6 (5.212)	94.1 (5.862)	96.2 (8.459)	93.4 (7.822)
CD45R	98.3 (47.09)	98.3 (51.06)	96.9 (38.22)	94.9 (34.11)
LFA-1	99.8 (7.895)	99.8 (9.076)	98.4 (6.571)	99.4 (6.914)
Mel-14	80.1 (7.963)	72.3 (6.936)	77.8 (5.494)	72.4 (3.853)

CD8$^+$ depleted spleen cells of Balb/cByJ mice were double stained with anti-Vβ8 (PE; red) and various antibodies specific for LFA-1, Ly-24, CD45R, and Mel-14 (recognizes homing receptor) (FITC; green). Data were presented as two-dimensional counter maps. The PE-stained CD4$^+$, Vβ8$^+$ T cell population was determined by software gating and FITC fluorescence was presented by a single parameter histogram. Data was shown by percent and mean of fluorescence (shown in brackets) positive cells. Data represent the average of triplicate samples.

Control* and SEB**: Spleen cells were prepared from PBS or 100 µg SEB-primed age matched thymectomized Balb/cByJ mice.

Table 6

Kinetics of Vβ8$^+$ T cells in SEB-tolerant thymectomized Balb/c mice.

Phenotype	Control**	10 days	2 months	4 months
	% positive cells*			
CD4$^+$, Vβ8$^+$	30.3	18.8	21.2	19.0
CD4$^+$, Vβ6$^+$	12.8	12.5	14.6	17.0
CD8$^+$, Vβ8$^+$	38.1	39.7	32.7	38.4
CD8$^+$, Vβ6$^+$	9.7	8.2	10.9	8.9

Data represent the average of triplicate samples.

% positive cells*: Vβ8$^+$ or Vβ6$^+$ cells were measured and their proportions in CD4$^+$ or CD8$^+$ T cells are calculated.

Control**: Age matched PBS-primed thymectomized mice were used as the control on each time point. The results were identical for each time point and therefore were represented by the values of 10 day PBS-primed mice.

Table 7

Treatment of C3H/He mice bearing 38C13 B lymphoma with SEB-anti-Id.

Treatment	Death	Mean survival (days) day ± SD*
PBS	14/14	19 ± 3
anti-Id	14/14	21 ± 3
SEB	14/14	20 ± 3
SEB + anti-Id	14/14	24 ± 6
SEB-anti-Id (conjugate)	8/14	>100

Groups of 14 C3H/He mice were injected with 38C13 cells intra-peritoneally (10^3/mouse in a final volume of 0.2 ml). Mice were treated once with indicated reagents intravenously 6 hrs after the tumor inoculation.

SD*: standard deviation

Table 8

Treatment of C3H/He mice bearing 38C13 B lymphoma with SEB-anti-Id, anti-CD3-anti-Id, and anti-Vβ8-anti-Id.

Treatment	Death	Mean survival (days) day ± SD
PBS	10/10	19 ± 3
anti-CD3-anti-Id (conjugate)	10/10	20 ± 3
SEB-anti-Id (conjugate)	6/10	>100
anti-Vβ8-anti-Id (conjugate)	7/10	>100

Groups of 10 C3H/He mice were injected with 38C13 cells intra-peritoneally (10^3/mouse in a final volume of 0.2 ml). Mice were treated once with 50 μg of indicated reagents intravenously 6 hrs after the tumor inoculation.

Table 9

Treatment of C3H/He mice bearing 38C13 B lymphoma* with SEB-anti-Id and anti-CD3-anti-Id.

Treatment	Death	Mean survival (days) day ± SD
PBS	8/8	20 ± 5
SEB	8/8	21 ± 3
anti-CD3	7/8	>100
anti-Id	6/8	>100
anti-CD3 + anti-Id	6/8	>100
anti-CD3-anti-Id (conjugate)	0/8	>100
SEB-anti-Id (conjugate)	0/8	>100

Groups of 8 C3H/He mice were injected with 38C13 cells intra-peritoneally (10^3/mouse in a final volume of 0.2 ml). Mice were treated once with 50 μg of indicated reagents intravenously 6 hrs after the tumor inoculation.

* An idiotype positive 38C13 subclone which grow slower than original cell (doubling time is about 1.5 times longer than that of wild type cell) was used in this experiment.

Table 10

Depletion of Vβ8⁺ T cells by anti-Vβ8 antibody but not by SEB treatment.

Treatment	Percent of positive cells	
	Vβ8	Vβ6
PBS	33.4±0.3	11.5±0.6
SEB	27.1±0.2	12.6±0.1
Anti-Vβ8	11.3±0.3	16.1±0.2

C3H/He mice were injected with 0.2 ml of SEB or anti-Vβ8 antibody (50 μg each), or PBS. Ten days later spleen single cell suspensions were studied for the percents of $V\beta6^+$ or $V\beta8^+$ T cells. Each value of the percent of positive cell indicated the proportion of $V\beta8^+$ or $V\beta6^+$ cells among $Thy1.2^+$ cells.

I CLAIM:

1. A method of treating autoimmune diseases associated with a predominance of T cells expressing Vβ8$^+$ T cell receptor comprising administering an amount of Staphylococcus enterotoxin B, a derivative, analogue or active fragment thereof, effective to inactivate and/or reduce the number of T cells expressing Vβ8$^+$ T cell receptor whereby there is a decrease in disease activity.

2. The method as claimed in claim 1, wherein the autoimmune diseases are multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, Type I diabetes, myasthenia gravis and pemphigus vulgaris.

3. A method of using Staphylococcus entertoxin B, derivatives, analogues or fragments thereof to assay for T cells expressing Vβ8$^+$ T cell receptor associated with autoimmune disease pathogenesis in a sample.

4. A method of using Staphylococcal aureus entertoxin B, derivatives, analogues or fragments thereof to down regulate lymphokines, preferably tumor necrosis factor and/or IL6.

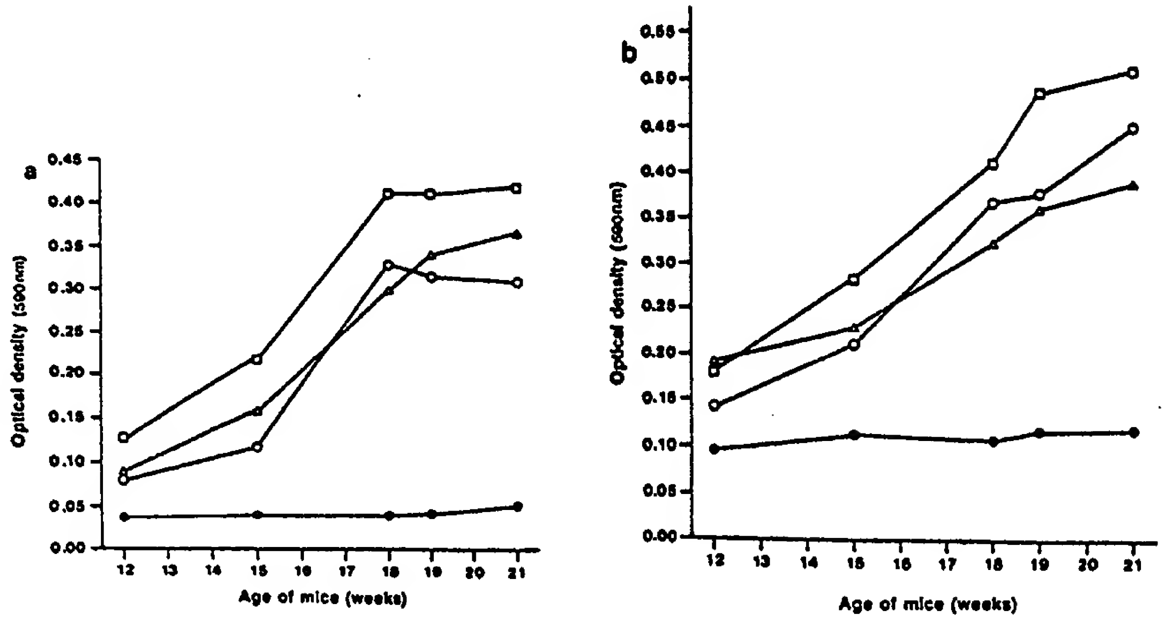
a
Optical density (590nm)
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0.40
0.45
12
13
14
15
16
17
18
19
20
21
Age of mice (weeks)
b
Optical density (590nm)
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0.40
0.45
0.50
0.55
Age of mice (weeks)

Figure 1

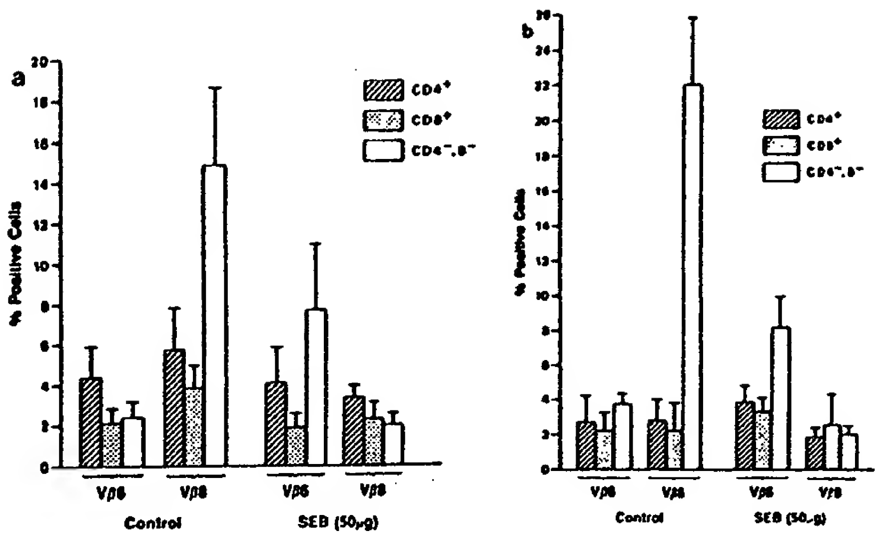

Figure 2

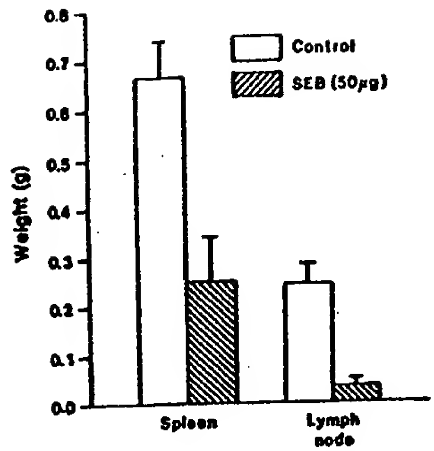
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Weight (g)
Control
SEB (50µg)
Spleen
Lymph node

Figure 3

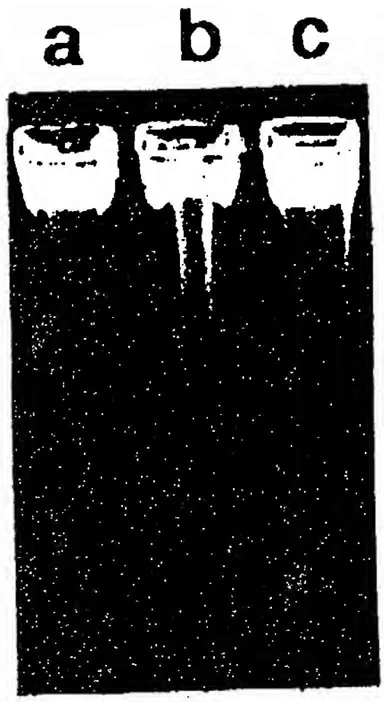

Figure 4

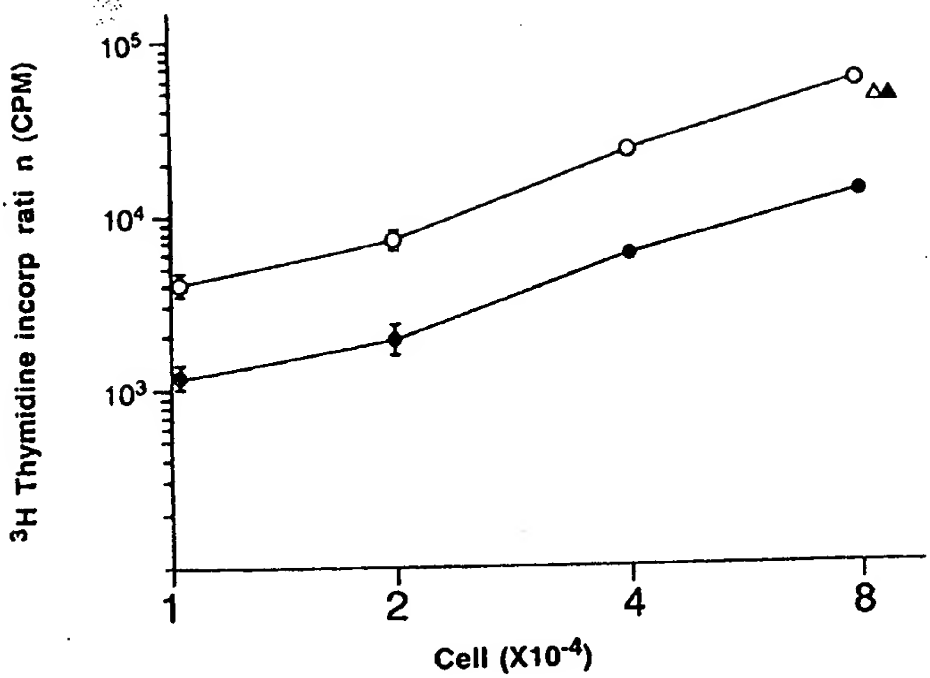

Figure 5

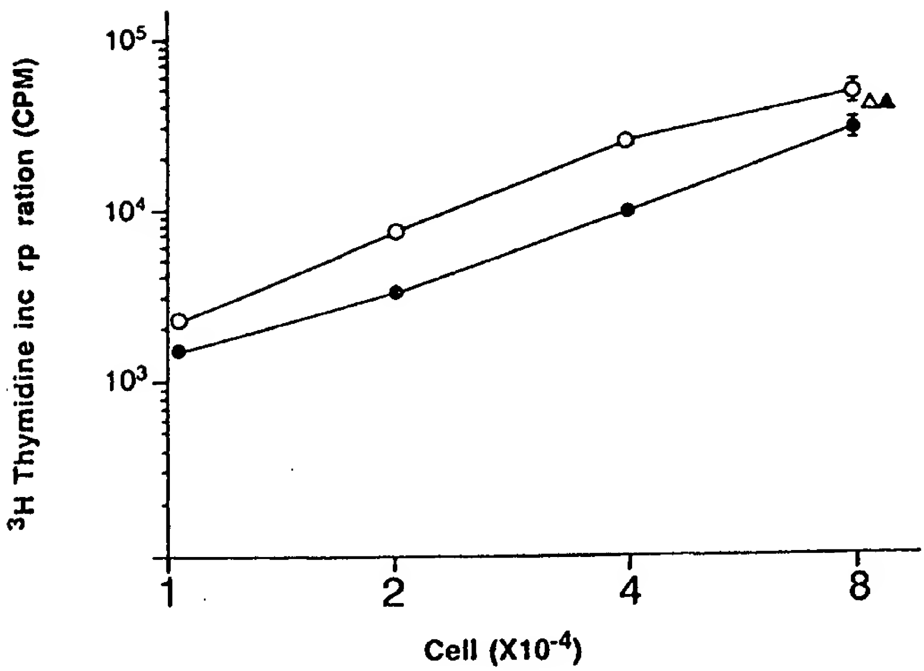

Figure 6

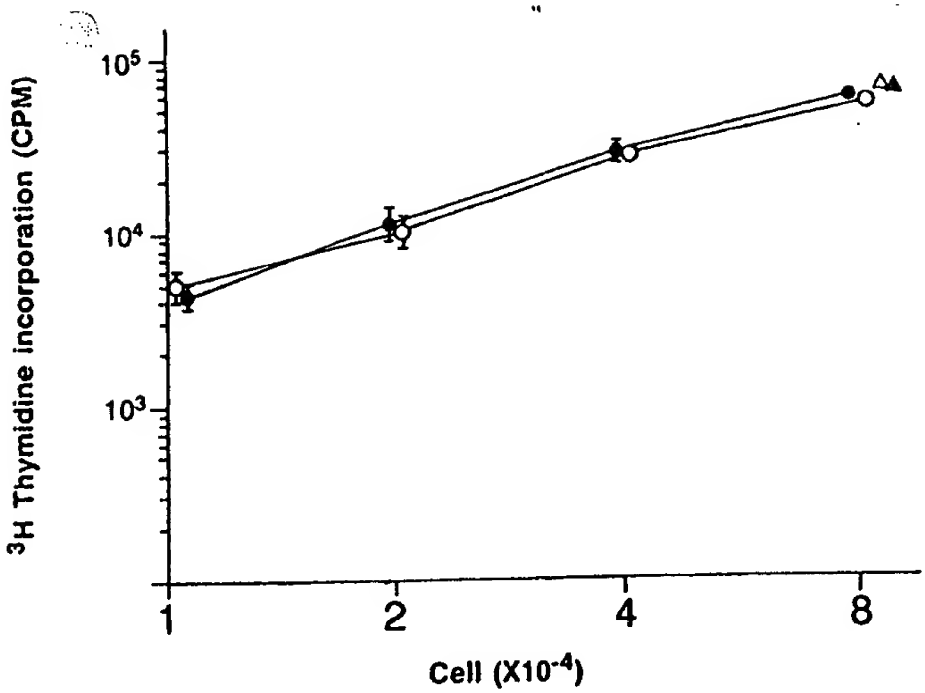

Figure 7A

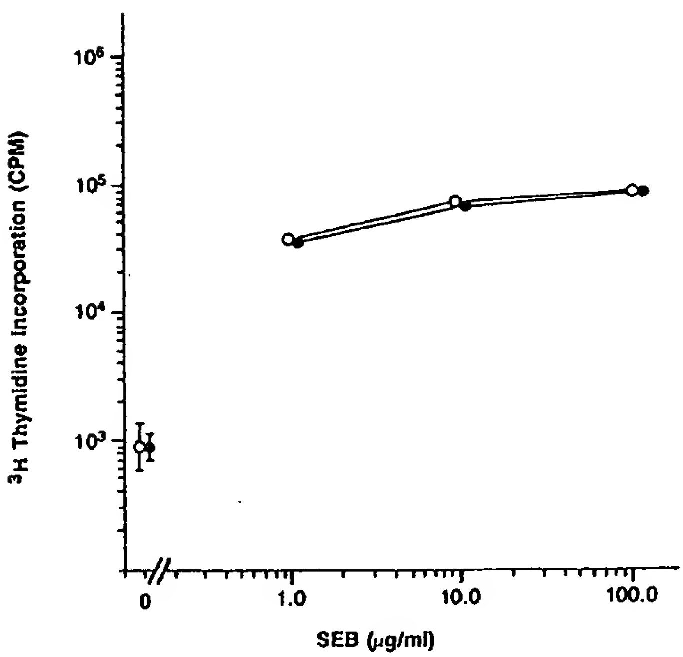

Figure 7B

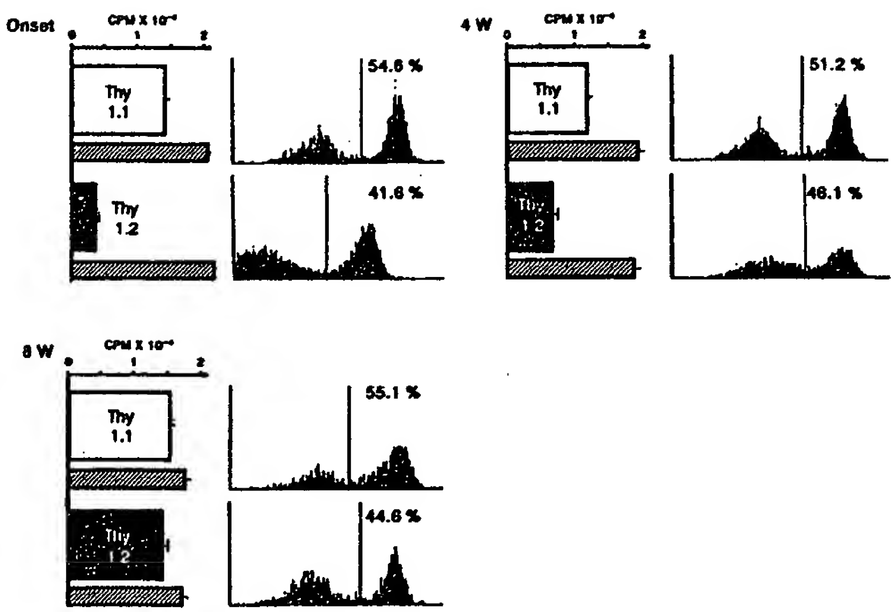
Onset
CPM X 10⁻⁴
0
1
2
Thy 1.1
54.6 %
Thy 1.2
41.6 %
4 W
CPM X 10⁻⁴
0
1
2
Thy 1.1
51.2 %
Thy 1.2
48.1 %
8 W
CPM X 10⁻⁴
0
1
2
Thy 1.1
55.1 %
Thy 1.2
44.6 %

Figure 8

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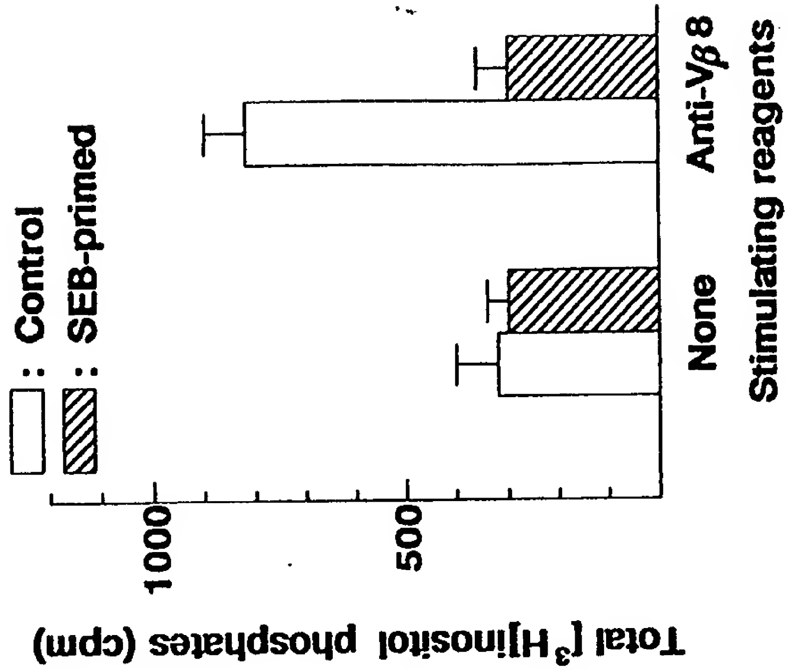


Figure 9A

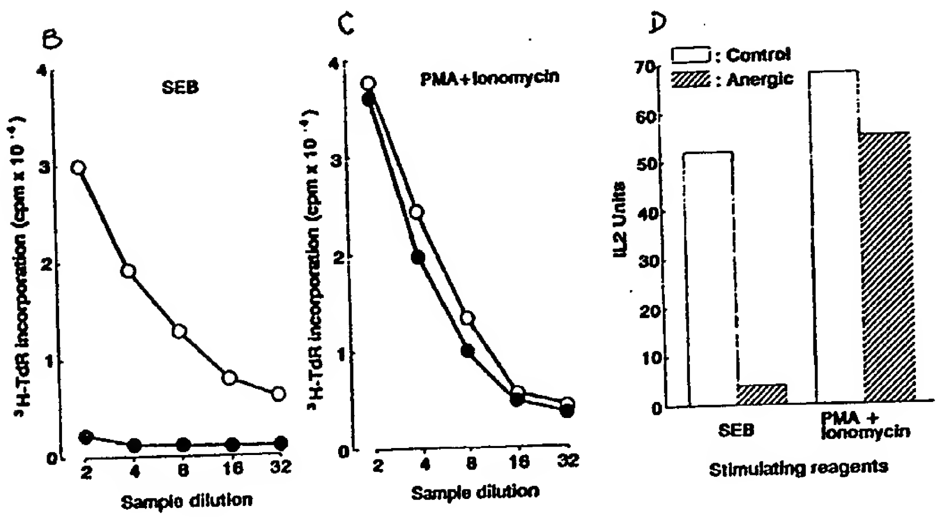


Figure 9B, 9C, 9D

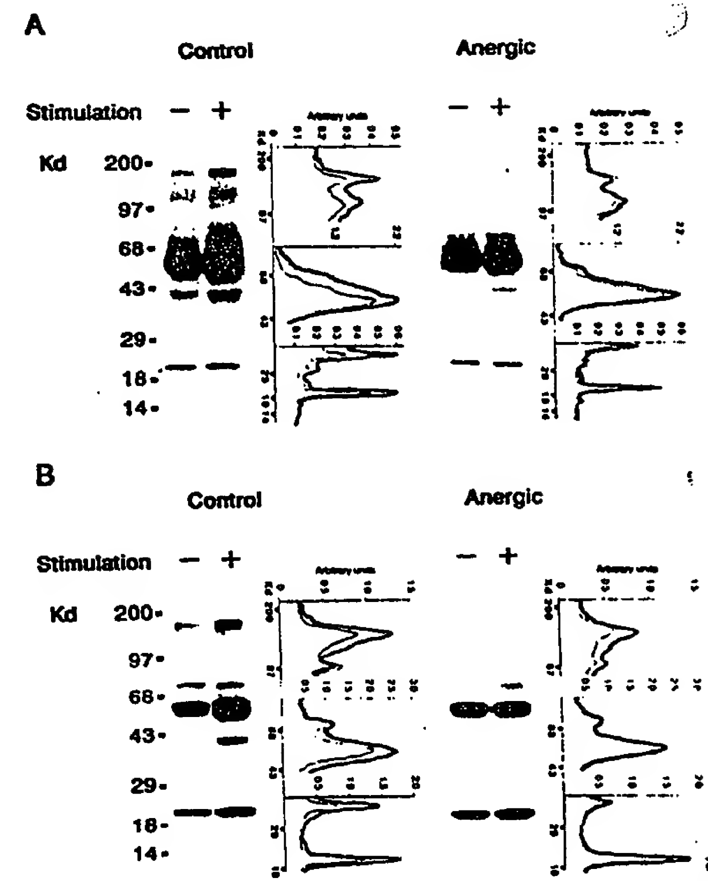


Figure 10

Figure 11A

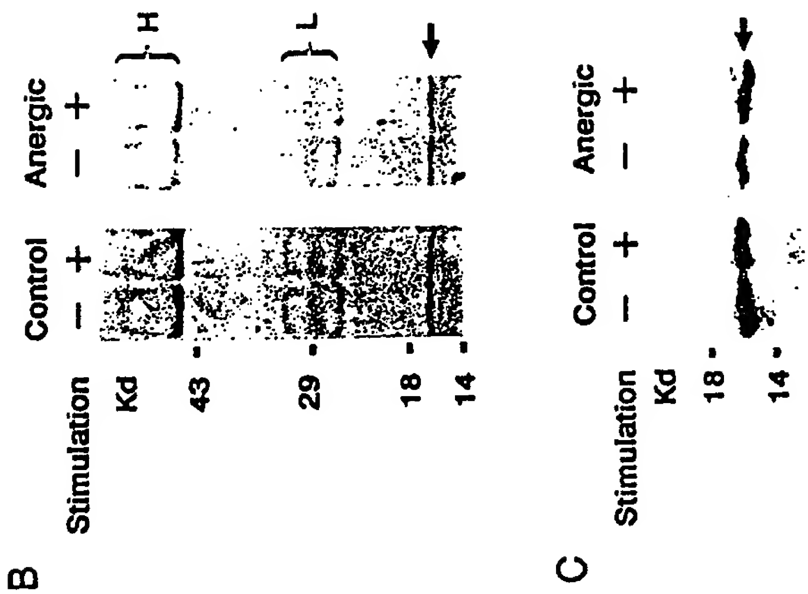


Figure 11B, 11C

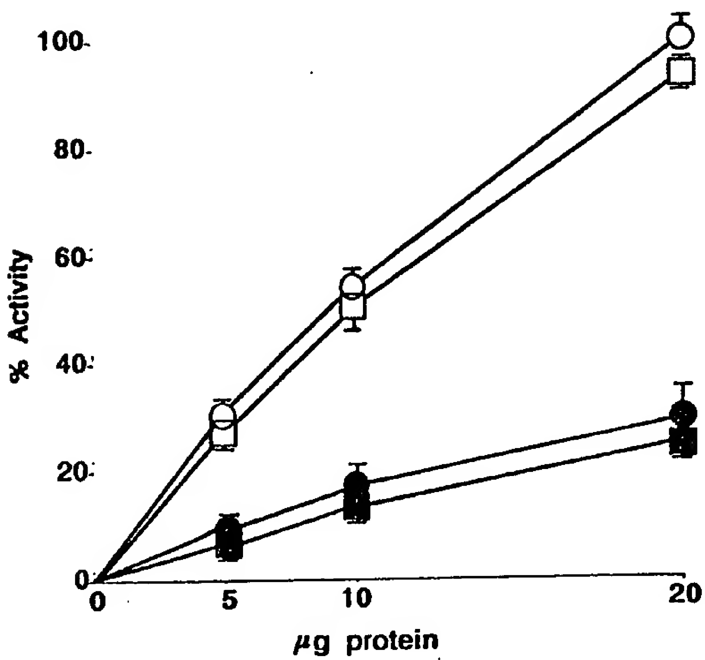


Figure 12

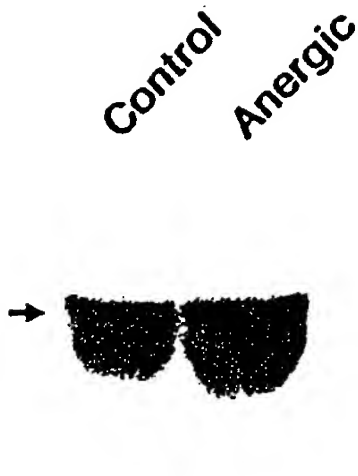


Figure 13A

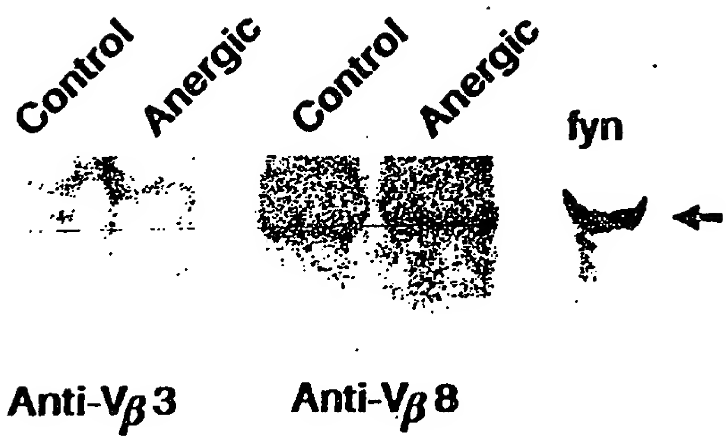


Figure 13B

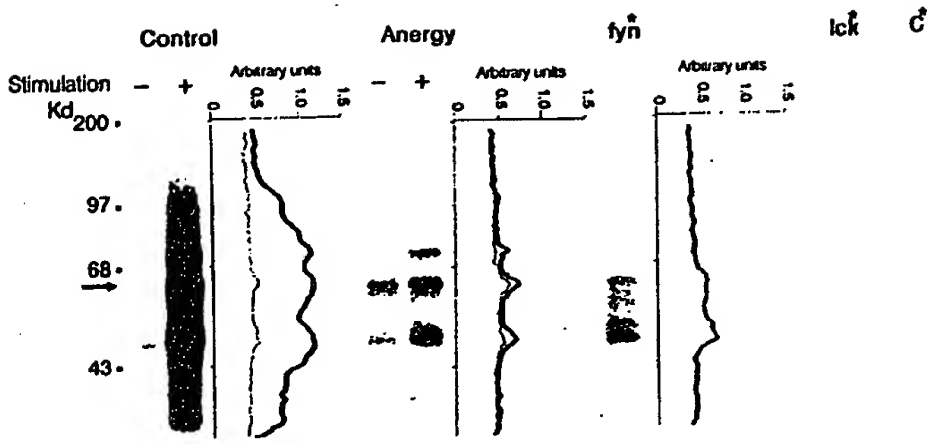

Control
Anergy
fyn*
lck*
c*
Stimulation
Kd
200
97
68
43
Arbitrary units
0
0.5
1.0
1.5

Figure 13C

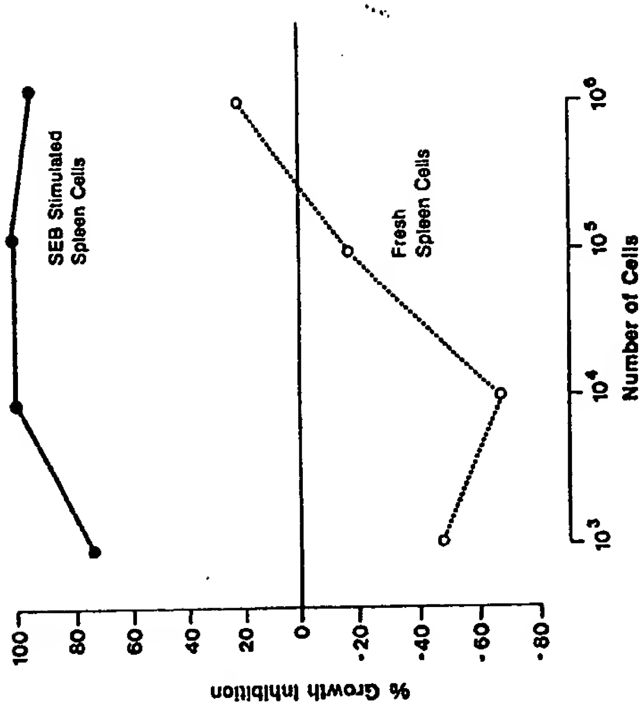


Figure 14A

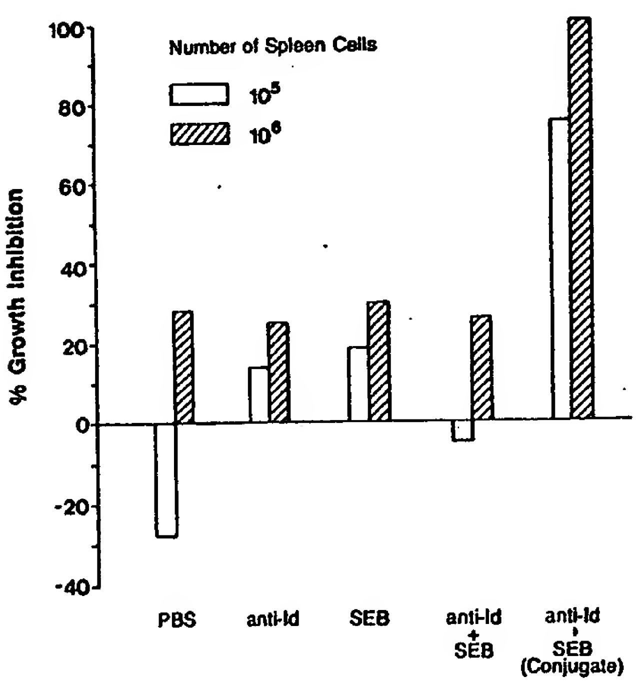
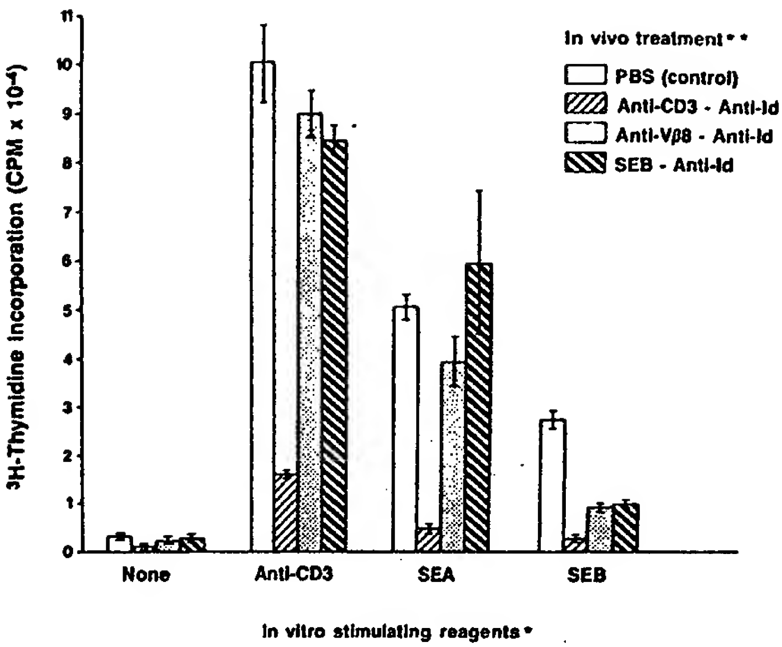


Figure 14B



3H-Thymidine Incorporation (CPM x 10-4)
0
1
2
3
4
5
6
7
8
9
10
11
In vivo treatment**
PBS (control)
Anti-CD3 - Anti-Id
Anti-Vβ8 - Anti-Id
SEB - Anti-Id
None
Anti-CD3
SEA
SEB
In vitro stimulating reagents*

Figure 15

2084120

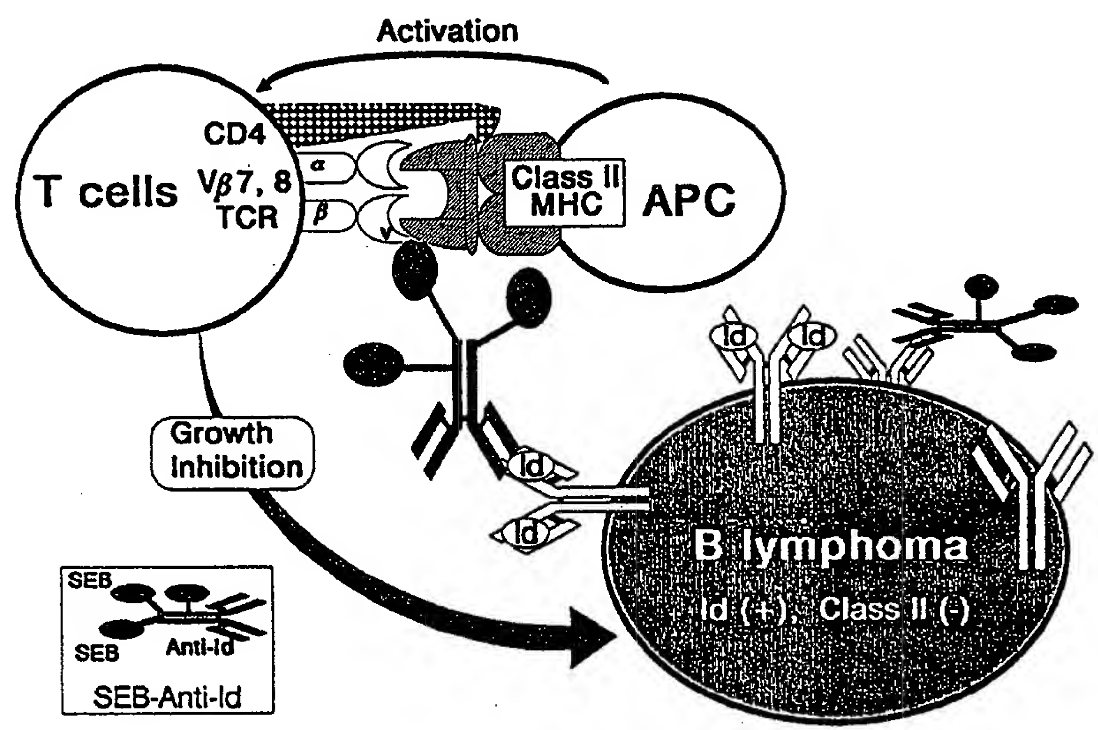
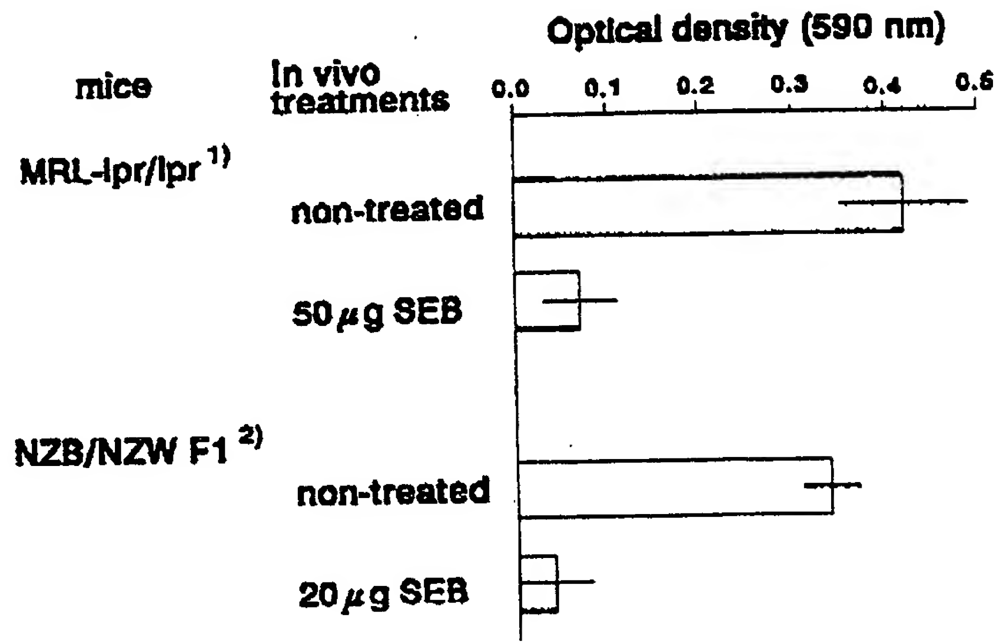


Figure 16

FIGURE 17


Optical density (590 nm)
mice
In vivo treatments
0.0
0.1
0.2
0.3
0.4
0.5
MRL-lpr/lpr 1)
non-treated
50 μg SEB
NZB/NZW F1 2)
non-treated
20 μg SEB